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| (54) Title: PROTEASE-CONTAINING CLEANING COMPOSITIONS | | |
| (57) Abstract The present invention relates to cleaning compositions comprising protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions correspond to naturally-occurring subtilisin from <u>Bacillus amyloliquefaciens</u> or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as <u>Bacillus lentus subtilisin</u>). | | |

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PROTEASE-CONTAINING CLEANING COMPOSITIONS

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This application is a continuation-in-part application of U.S. Application
15 Serial No. 08/136,797, filed October 14, 1993 and U.S. Application Serial No.
08/237,938, filed May 2, 1994, both incorporated herein by reference in their
entirety.

TECHNICAL FIELD

The present invention relates to a variety of cleaning compositions
20 comprising novel protease enzymes which are carbonyl hydrolase variants.

BACKGROUND

Enzymes make up the largest class of naturally occurring proteins.
Each class of enzyme generally catalyzes (accelerates a reaction without being
consumed) a different kind of chemical reaction. One class of enzymes, known
25 as proteases, are known for their ability to hydrolyze (break down a compound
into two or more simpler compounds with the uptake of the H and OH parts of a
water molecule on either side of the chemical bond cleaved) other proteins.
This ability to hydrolyze proteins has been taken advantage of by incorporating
naturally occurring and protein engineered proteases as an additive to laundry
30 detergent preparations. Many stains on clothes are proteinaceous and wide-
specificity proteases can substantially improve removal of such stains.

Unfortunately, the efficacy level of these proteins in their natural,
bacterial environment, frequently does not translate into the relatively unnatural
wash environment. Specifically, protease characteristics such as thermal
35 stability, pH stability, oxidative stability and substrate specificity are not
necessarily optimized for utilization outside the natural environment of the
enzyme.

The amino acid sequence of the protease enzyme determines the characteristics of the protease. A change of the amino acid sequence of the protease may alter the properties of the enzyme to varying degrees, or may even inactivate the enzyme, depending upon the location, nature and/or
5 magnitude of the change in the amino acid sequence. Several approaches have been taken to alter the amino acid sequence of proteases in an attempt to improve their properties, with the goal of increasing the efficacy of the protease for cleaning uses such as in the wash environment. These approaches include altering the amino acid sequence to enhance thermal stability and to improve
10 oxidation stability under quite diverse conditions.

Despite the variety of approaches described in the art, there is a continuing need for new effective variants of proteases useful for cleaning a variety of surfaces. It is therefore an object of the present invention to provide cleaning compositions containing protease enzymes which are carbonyl
15 hydrolase variants having improved proteolytic activity, substrate specificity, stability and/or enhanced performance characteristics. These and other objects will become readily apparent from the detailed description which follows.

SUMMARY OF THE INVENTION

20 The present invention relates to cleaning compositions comprising:
(a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid
25 residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions corresponds to naturally-occurring subtilisin from
30 Bacillus amyloliquefaciens or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as Bacillus lentus subtilisin); and
(b) one or more cleaning composition materials compatible with the protease enzyme.

The present invention also relates to methods for cleaning items in need
35 of cleaning by contacting said item with a protease enzyme which is a carbonyl hydrolase variant as described herein. The invention therefore encompasses a method for cleaning fabrics comprising contacting, preferably with agitation,

said fabrics with an aqueous liquor containing said protease enzyme. The method can be carried out at temperatures below about 60°C but, of course, is quite effective at laundry temperatures up to the boil. The present invention also relates to a method for cleaning dishes by contacting a dish in need of
5 cleaning with a protease enzyme as described herein. The present invention methods also include methods for personal cleansing, said methods comprising contacting the part of the human or lower animal body in need of cleaning with a protease enzyme as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No.6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN') and *Bacillus lentus* (wild-type).

15 Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No.7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid
20 sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

25 Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No.11). The mature subtilisin protein is coded by
30 the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103A/V104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The
35 mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

Fig. 10 shows the stability of a preferred mutant enzyme compared to wild-type, in a liquid detergent formulation.

DETAILED DESCRIPTION OF THE INVENTION

5 1. Protease Enzymes:

The invention includes protease enzymes which are non-naturally-occurring carbonyl hydrolase variants having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid
10 sequence of the variant is derived. The precursor carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. The plurality of
15 amino acid residues of the precursor enzyme correspond to position +76 in combination with one or more of the following residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered position corresponds to naturally-occurring subtilisin from
20 *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins, such as *Bacillus lentus* subtilisin.

The carbonyl hydrolase variants which are protease enzyme useful in the present invention compositions comprise replacement of amino acid residue +76 in combination with one or more additional modifications.
25 Preferably the variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104;
30 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably
35 the variant nzymes us ful for th pr sent invention comprise th substitution, deletion or insertion of an amino acid residue in the following combination of

residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104 of *B. amyloliquefaciens* subtilisin.

Variant DNA sequences encoding such carbonyl hydrolase or subtilisin variants are derived from a precursor DNA sequence which encodes a naturally-occurring or recombinant precursor enzyme. The variant DNA sequences are derived by modifying the precursor DNA sequence to encode the substitution of one or more specific amino acid residues encoded by the precursor DNA sequence corresponding to positions 76, 99, 101, 103, 104, 107, 123, 27, 105, 109, 126, 128, 135, 156, 166, 195, 197, 204, 206, 210, 216, 217, 218, 222, 260, 265 and/or 274, in *Bacillus amyloliquefaciens* or any combination thereof. Although the amino acid residues identified for modification herein are identified according to the numbering applicable to *B. amyloliquefaciens* (which has become the conventional method for identifying residue positions in all subtilisins), the preferred precursor DNA sequence useful for the present invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 6 (Seq. ID No. 11).

These variant DNA sequences encode the insertion or substitution of the amino acid residue 76 in combination with one or more additional modification. Preferably the variant DNA sequences encode the substitution or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably the variant DNA sequences encode for the modification of the following combinations of residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These recombinant DNA sequences encode carbonyl hydrolase variants having a novel amino acid sequence and, in general, at least one property which is substantially different from the same property of the enzyme encoded by the precursor carbonyl hydrolase DNA sequence. Such properties include proteolytic activity, substrate specificity, stability, altered pH profile and/or enhanced performance characteristics.

Th protease nzymes useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application
 5 reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, J.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

Preferably, the substitution to be made at each of the identified amino acid residue positions include but are not limited to: substitutions at position
 10 76 including D, H, E, G, F, K, P and N; substitutions at position 99 including D, T, N, Q, G and S; substitutions at position 101 including G, D, K, L, A, E, S and R; substitutions at position 103 including Q, T, D, E, Y, K, G, R, S, and A; substitutions at position 104 including all nineteen naturally-occurring amino acids; substitutions at position 107 including V, L, M, Y, G, E, F, T, S, A, N and
 15 I; substitutions at position 123 including N, T, I, G, A, C, and S; substitutions at position 27 including K, N, C, V and T; substitutions at position 105 including A, D, G, R and N; substitutions at position 107 including A, L, V, Y, G, F, T, S and A; substitutions at position 109 including S, K, R, A, N and D; substitutions at position 126 including A, F, I, V and G; substitutions at position 128 including
 20 G, L and A; substitutions at position 135 including A, F, I, S and V; substitutions at position 156 including D, E, A, G, Q and K; substitutions at position 166 including all nineteen naturally-occurring amino acids; substitutions at position 195 including E; substitutions at position 197 including E; substitutions at position 204 including A, G, C, S and D; substitutions at position 206 including
 25 L, Y, N, D and E; substitutions at position 210 including L, I, S, C and F; substitutions at position 216 including V, E, T and K; substitutions at position 217 including all nineteen naturally-occurring amino acids; substitutions at position 218 including S, A, G, T and V; substitutions at position 222 including all nineteen naturally-occurring amino acids; substitutions at position 260
 30 including P, N, G, A, S, C, K and D; substitutions at position 265 including N, G, A, S, C, K, Y and H; and substitutions at position 274 including A and S. The specifically preferred amino acid(s) to be substituted at each such position are designated below in Table I. Although specific amino acids are shown in Table I, it should be understood that any amino acid may be substituted at the
 35 identified residues.

Table I

Amino Acid

Preferred Amino Acid to

| | <u>R sidue</u> | <u>b Substituted/Insert d</u> |
|----|----------------|-------------------------------|
| | +76 | D,H |
| | +99 | D,T,N,G |
| | +101 | R,G,D,K,L,A,E |
| 5 | +103 | A,Q,T,D,E,Y,K,G,R |
| | +104 | I,Y,S,L,A,T,G,F,M,W,D,V,N |
| | +107 | V,L,Y,G,F,T,S,A,N |
| | +123 | S,T,I |
| | +27 | K |
| 10 | +105 | A,D |
| | +109 | S,K,R |
| | +126 | A,I,V,F |
| | +128 | G,L |
| | +135 | I,A,S |
| 15 | +156 | E,D,Q |
| | +166 | D,G,E,K,N,A,F,I,V,L |
| | +195 | E |
| | +197 | E |
| | +204 | A,G,C |
| 20 | +206 | L |
| | +210 | I,S,C |
| | +216 | V |
| | +217 | H,I,Y,C,A,G,F,S,N,E,K |
| | +218 | S |
| 25 | +222 | A,Q,S,C,I,K |
| | +260 | P,A,S,N,G |
| | +265 | N,A,G,S |
| | +274 | A,S |

These protease enzymes containing *in vitro* mutations in *B. lentus* subtilisin at an amino acid residue equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin produces subtilisin variants exhibiting altered stability (e.g., modified autoproteolytic stability) over precursor subtilisins. (See Tables IV and VI.)

Also, *in vitro* mutation at residues equivalent to +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin, alone or in combination with each other

and in any combination with +76 mutations, produce subtilisin variants exhibiting altered proteolytic activity, altered thermal stability, altered pH profile, altered substrate specificity and/or altered performance characteristics.

5 Carbonyl hydrolases are protease enzymes which hydrolyze compounds containing



10 bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, 15 carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, 20 insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258, the disclosure of which are incorporated herein by reference.

Subtilisins are bacterial or fungal carbonyl hydrolases which generally 25 act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the 30 same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related 35 proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however, is histidine-aspartate-serine. Thus,

subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein.

"Recombinant subtilisin" refers to a subtilisin in which the DNA
5 sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in U.S. Patent 4,760,025
10 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*.
15 Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, *bovine* sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases
20 can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or
25 indirectly, with procaryotic and eucaryotic sources.

A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases (such as a subtilisin) include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases
30 (subtilisin). The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase
35 (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as

meth ds kn wn to those skilled in the art (s e, for xampl , EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific residues corresponding to position +76 in combination with one or more of the following positions +99, +101, +103, +104, +107, +123, +27,
 5 +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 of *Bacillus amyloliquefaciens* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104;
 10 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126;
 15 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222; and most preferably are 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The protease
 20 enzymes useful in the present invention, however, are not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. Preferably, the precursor subtilisin is *Bacillus lentus* subtilisin and the
 25 substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to
 30 a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *Bacillus*
 35 *amyloliquefaciens* subtilisin primary s quenc and particularly to a s t f residues known to be invariant in subtilisins for which sequence is known. Fig. 2 herein shows the conserved residues as between *amyloliquefaciens* subtilisin

and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of
5 *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved
residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

10 For example, in Fig. 3 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in
15 each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lentus*
20 (PCT Publication No. W089/06279 published July 13, 1989), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP O 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce
25 the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

30 Thus, for example, the amino acid at position +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of all the amino acid residues identified herein for substitution
35 versus the preferred substitution for each such position is provided in Table II for illustrative purposes.

Tabl II

| | <u>+76</u> | <u>+99</u> | <u>+101</u> | <u>+103</u> | <u>+104</u> | <u>+107</u> | <u>+123</u> |
|--|------------|------------|-------------|-------------|-------------|-------------|-------------|
| <i>B. amyloliquefaciens</i> (wild-type) | N | D | S | Q | Y | I | N |
| <i>B. lentus</i> (wild-type) | N | S | S | S | V | I | N |

Most Preferred Substitution D D R A I/Y V S

Equivalent residues may also be defined by determining homology at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to U.S. Patent Application SN

08/212,291, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

5 Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring
10 sequence. The carbonyl hydrolase variants useful in the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

15 "Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are
20 expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In the Examples, the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) is used.

25 A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by
30 the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The protease enzymes useful for the present invention utilize such sequences to effect the secretion of the carbonyl hydrolase variants as described herein. A preferred signal
35 sequence used in the Examples comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

5 "Expression vector" refers to a DNA construct containing a DNA
sequence which is operably linked to a suitable control sequence capable of
effecting the expression of said DNA in a suitable host. Such control
sequences include a promoter to effect transcription, an optional operator
sequence to control such transcription, a sequence encoding suitable mRNA
10 ribosome binding sites and sequences which control termination of
transcription and translation. The vector may be a plasmid, a phage particle, or
simply a potential genomic insert. Once transformed into a suitable host, the
vector may replicate and function independently of the host genome, or may, in
some instances, integrate into the genome itself. In the present specification,
15 "plasmid" and "vector" are sometimes used interchangeably as the plasmid is
the most commonly used form of vector at present. However, included herein
are such other forms of expression vectors which serve equivalent functions
and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic
20 or eucaryotic hosts which preferably have been manipulated by the methods
disclosed in U.S. Patent 4,760,025 (RE 34,606) to render them incapable of
secreting enzymatically active endoprotease. A preferred host cell for
expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in
enzymatically active neutral protease and alkaline protease (subtilisin). The
25 construction of strain BG2036 is described in detail in U.S. Patent 5,264,366.
Other host cells for expressing subtilisin include *Bacillus subtilis* 1168 (also
described in U.S. Patent 4,760,025 (RE 34,606) and U.S. Patent 5,264,366, the
disclosure of which are incorporated herein by reference), as well as any
suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

30 Host cells are transformed or transfected with vectors constructed using
recombinant DNA techniques. Such transformed host cells are capable of
either replicating vectors encoding the carbonyl hydrolase variants or
expressing the desired carbonyl hydrolase variant. In the case of vectors
which encode the pre- or prepro-form of the carbonyl hydrolase variant, such
35 variants, when expressed, are typically secreted from the host cell into the host
cell medium.

"Operably linked," when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein
5 most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl
10 hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes.
15 Positively hybridizing clones are then mapped and sequenced. The *B. lentus* gene used in the Examples is cloned as described in Example 1 of U.S. Patent 5,185,258, the disclosure of which is incorporated herein. The BPN' gene used in the Examples is cloned as described in Example 1 in RE 34,606, the disclosure of which is incorporated herein.

20 The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the
25 gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the hydrolase gene and, desirably, a selection gene such
30 as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate
35 multiple copies of the hydrolase gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

Th genes used in th present xampl s are a natural *B. lentus* g n and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid
5 sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of U.S. Patent 5,204,015, the disclosure of which is
10 incorporated herein by reference.

Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant
15 carbonyl hydrolases as disclosed in U.S. Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants useful in the
20 present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The
25 sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site
30 which is not overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such
35 sit s are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amin acids ncoded ar changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in

accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different
5 restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the
10 cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

15 As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant
20 enzymes of the present invention may have other modified properties such as K_m , K_{cat} , K_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, for hydrolytic processes such as laundry uses.

25 One objective can be to secure a variant carbonyl hydrolase having altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are variant enzymes having altered thermal stability and/or altered substrate specificity as
30 compared to the precursor. Preferably the carbonyl hydrolase to be mutated is a subtilisin. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 or any
35 combination thereof in *Bacillus amyloliquefaciens* subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. Conversely, in some instances it may be desirable to increase the proteolytic

activity of the variant enzyme versus its precursor. Additionally, increases or decreases (alteration) of the stability of the variant, whether alkaline or thermal stability, may be desirable. Increases or decreases in K_{cat} , K_m or K_{cat}/K_m are specific to the substrate used to determine these kinetic parameters.

5 Also, it has been determined that residues equivalent to +76 in combination with a number of other modifications in subtilisin are important in modulating overall stability and/or proteolytic activity of the enzyme. Thus, as set forth in the Examples, the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 can be substituted with Aspartate (D) in the preferred
10 protease enzymes in combination with modification of one or more of the following amino acid residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

15 The most preferred protease enzymes useful in this invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/S99D; N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V and N76D/S101R/S103A/V104I. These substitutions are preferably made in
20 *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* subtilisin.

Based on the results obtained with this and other variant subtilisins, it is apparent that residues in carbonyl hydrolases (preferably subtilisin) equivalent to positions +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126,
25 +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in *Bacillus amyloliquefaciens* are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

The following is presented by way of example for manufacturing
30 protease enzymes useful in the present invention compositions.

Protease Manufacture Example

Construction for the Expression of GG36 Gene in *B. subtilis*

The cloning and the construction for expression of the subtilisin gene from *B. lentus* is performed essentially the same as that described in U.S. Patent
35 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) is further modified in the following manner, as shown in Fig. 5. The PstI site that is introduced during the construction of the GGA274 plasmid is removed by th

oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5' GAAGCTGCAACTCGTTAAA 3' (Seq. ID No.1). The underlined "A" residue eliminates the recognition sequence of restriction enzyme PstI and changes the corresponding amino acid residue from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lentus* subtilisin gene sequences. The DNA segment encoding subtilisin is excised from the plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment is subcloned back into Bacteriophage M13-based vectors, such as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, are performed to move the mutated subtilisin gene back into an expression plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

Oligonucleotide-Directed Mutagenesis

Oligonucleotide-directed mutagenesis is performed as described in Zoller, M. et al. (1983), Methods Enzymol., 100:468-500. As an example, a synthetic oligonucleotide of the sequence 5' GCTGCTCTAGACAATTCG 3' (Seq. ID No.2) is used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC changes asparagine at +76 to aspartate.

For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the combination of mutations desired. For example, an oligonucleotide of the sequence 5' GTATTAGGGGCGGACGGTCGAGGCCATCAGCTCGATT 3'(Seq. ID No.3) is used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the sequence 5' TCAGGTTCGGTCTCGAGCGTTGCCCAAGGATTG 3' (Seq. ID No.4) and 5' CACGTTGCTAGCTTGAGTTTAG 3' (Seq. ID No.5) are utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produce desired changes either in amino acid sequences or restriction enzyme recognition sequences.

Proteolytic Activity of Subtilisin Variants

Following the methods of Oligonucleotide-Directed Mutagenesis hereinbefore, the variants listed in Table III are made. Proteolytic activity of

each of these subtilisin variants is shown in Table III. The kinetic parameters k_{cat} , K_M , and k_{cat}/K_M are measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p. 1030. Briefly, a small aliquot of subtilisin variant stock solution is added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at 25°C. The reaction progress is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm. Kinetic parameters are obtained by using a non-linear regression algorithm to fit the reaction velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table III

Kinetic Parameters k_{cat} , K_M and k_{cat}/K_M
Measured for Bacillus lentus Subtilisin and Variants

| <u>Protease #</u> | <u>Enzyme</u> <u>Variants</u> | <u>k_{cat} (s^{-1})</u> | <u>K_M (M)</u> | <u>k_{cat}/K_M</u> <u>($s^{-1}M^{-1}$)</u> |
|-------------------|-----------------------------------|---|-----------------------------|---|
| - | <u>B. lentus</u> Subtilisin | 170 | 0.00078 | 2.18×10^5 |
| - | N76D | 219 | 0.0008 | 2.74×10^5 |
| 1 | N76D/S99D | 88 | 0.00061 | 1.44×10^5 |
| 2 | N76D/S101R | 371 | 0.0013 | 2.85×10^5 |
| 3 | N76D/S103A | 400 | 0.0014 | 2.86×10^5 |
| 4 | N76D/V104I | 459 | 0.0011 | 4.17×10^5 |
| 5 | N76D/I107V | 219 | 0.0011 | 1.99×10^5 |
| 6 | N76D/N123S | 115 | 0.0018 | 6.40×10^4 |
| 7 | N76D/S99D/S101R | 146 | 0.00038 | 3.84×10^5 |
| 8 | N76D/S99D/S103A | 157 | 0.0012 | 1.31×10^5 |
| 9 | N76D/S99D/V104I | 247 | 0.00097 | 2.55×10^5 |
| 10 | N76D/S101R/S103A | 405 | 0.00069 | 5.90×10^5 |
| 11 | N76D/S101R/V104I | 540 | 0.00049 | 1.10×10^6 |
| 12 | N76D/S103A/V104I | 832 | 0.0016 | 5.20×10^5 |
| 13 | N76D/V104I/I107V | 497 | 0.00045 | 1.10×10^6 |
| 14 | N76D/V104Y/I107V | 330 | 0.00017 | 1.90×10^6 |
| 15 | N76D/V104I/N123S | 251 | 0.0026 | 9.65×10^4 |
| 16 | N76D/I107V/N123S | 147 | 0.0035 | 4.20×10^4 |
| 17 | N76D/S99D/S101R/S103A | 242 | 0.00074 | 3.27×10^5 |
| 18 | N76D/S99D/S101R/V104I | 403 | 0.00072 | 5.60×10^5 |
| 19 | N76D/S99D/S103A/V104I | 420 | 0.0016 | 2.62×10^5 |
| 20 | N76D/S101R/S103A/V104I | 731 | 0.00065 | 1.12×10^6 |
| 21 | N76D/S103A/V104I/N123S | 321 | 0.0026 | 1.23×10^5 |
| 22 | N76D/V104I/I107V/N123S | 231 | 0.003 | 7.70×10^4 |
| 23 | N76D/S99D/S101R/S103A/V104I | 624 | 0.00098 | 6.37×10^5 |
| 24 | N76D/S99D/S103A/V104I/N123S | 194 | 0.0043 | 4.51×10^4 |
| 25 | N76D/S99D/S101R/S103A/V104I/N123S | 311 | 0.0023 | 1.35×10^5 |

The results listed in Table III indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal

5 that proteolytic activity is significantly altered for *Bacillus lentus* subtilisin by the various combinations of substitutions at amino acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and 123 in *Bacillus amyloliquefaciens*.

Thermal Stability of Subtilisin Variants

A comparison of thermal stability observed for *Bacillus lentus* subtilisin and the variants of the present invention made by the process of Oligonucleotide-Directed Mutagenesis hereinbefore is shown in Table IV. Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or without 50 mM CaCl₂, is aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes are then placed on ice for 10 minutes. Aliquots from the tubes are assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table IV indicate that a vast majority of variants exhibit thermal stability comparable to *Bacillus lentus* subtilisin (24 out of 26) in the test condition with 50mM CaCl₂ added. In the test condition without 50mM CaCl₂ added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lentus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM CaCl₂ added.

Table IV

Thermal Stability Measured for *Bacillus lentus* Subtilisin and Variants

At pH 10, 60°C, +/- 50mM CaCl₂ Added

| Enzyme | <u>% Initial Activity Remaining</u> | |
|-----------------------------|-------------------------------------|--------------------------|
| | <u>-CaCl₂</u> | <u>+CaCl₂</u> |
| <u>B. lentus</u> Subtilisin | 2 | 96 |
| N76D | 34 | 97 |
| N76D/S99D | 49 | 98 |
| N76D/S101R | 0 | 82 |
| N76D/S103A | 26 | 92 |
| N76D/V104I | 58 | 98 |
| N76D/I107V | 32 | 96 |
| N76D/N123S | 0 | 97 |
| N76D/S99D/S101R | 30 | 100 |
| N76D/S99D/S103A | 36 | 100 |
| N76D/S99D/V104I | 48 | 97 |

| | | |
|-----------------------------------|----|-----|
| N76D/S101R/S103A | 26 | 100 |
| N76D/S101R/V104I | 38 | 100 |
| N76D/S103A/V104I | 58 | 100 |
| N76D/V104I/I107V | 60 | 97 |
| N76D/V104Y/I107V | 48 | 74 |
| N76D/V104I/N123S | 0 | 98 |
| N76D/I107V/N123S | 16 | 100 |
| N76D/S99D/S101R/S103A | 38 | 100 |
| N76D/S99D/S101R/V104I | 33 | 100 |
| N76D/S99D/S103A/V104I | 38 | 98 |
| N76D/S101R/S103A/V104I | 40 | 99 |
| N76D/S103A/V104I/N123S | 1 | 98 |
| N76D/V104I/I107V/N123S | 3 | 99 |
| N76D/S99D/S101R/S103A/V104I | 36 | 99 |
| N76D/S99D/S103A/V104I/N123S | 2 | 95 |
| N76D/S99D/S101R/S103A/V104I/N123S | 0 | 100 |

Oligonucleotide-Directed Mutagenesis with
Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lentus* Variants

- The mutagenesis protocol is essentially the same as described above in
- 5 Oligonucleotide-Directed Mutagenesis. The single-stranded DNA template is generated by phagemid method. To construct the phagemid vector for generating the single-stranded DNA template we first construct the vector pBCDAICAT. The flow chart of vector construction is outlined in Figure 8. First, the *C1aI* to *C1aI* fragment encoding the CAT gene from pC194 plasmid
 - 10 (*Horinouchi, S. and Weisblum, B., J. Bacteriol.*, 150:8-15, 1982) is cloned into the *AccI* site of polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make plasmid pUCCHL and the *EcoRI-DraI* 0.6 KB fragment from the 5' end of the GG36DAI encoding DNA is cloned into the *EcoRI* and *EcoRV* sites of pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The
 - 15 single *EcoRI* site of the plasmid pBC2SK5 is eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI-DraI* fragment which is cloned into pBCSK-5R is isolated as a *PstI-HindIII* fragment and cloned into the *PstI-HindIII* site of the pUCCHL (part of the
 - 20 polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene is excised as an *EcoRI-BamHI* fragment and

cloned into the *EcoRI*-*BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI*-*HindIII* fragment of pUCCAT is then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

To generate single-stranded DNA, *E. coli*-containing pBCDAICAT are infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE 45:333-338, 1986. Once the single-stranded DNA template is available, standard mutagenesis methods as described above in Oligonucleotide-Directed Mutagenesis are carried out. The construction of certain mutants is detailed below for illustrative purposes.

For the construction of *B. lentus* (GG36) N76D/S103A/V104I/L217H, an *EcoRI*-*BamHI* DNA fragment encoding GG36 N76D/S103A/V104I is used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT. After the single-stranded DNA template is made following the protocol described above, a mutagenesis primer with the following sequence

* *** ** x *C1aI*.

5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)

is used to make the L217H. As before, the underlined residues denote the nucleotide changes that are made and the x *C1aI* indicates that the existing *C1aI* site is eliminated after the mutagenesis. The mutagenesis protocol is as described in Oligonucleotide-Directed Mutagenesis hereinbefore. After the mutagenesis, plasmid DNA is first screened for the elimination of the *C1aI* site and those clones missing the *C1aI* site are subjected to DNA sequence analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

B. Construction of BPN' Variants and their Expression in *B. subtilis*

The construction of *B. amyloliquefaciens* (BPN')

N76D/Q103A/Y104I/Y217L is done in a similar fashion except in two consecutive steps. N76D is first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis is done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI*-*BamHI* fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., PNAS, 84, 5167, 1087) is used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L is used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA is generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

* * * * * *Xba*I

5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3' (Seq. ID No.14)

- is used to generate the change N76D. Single-stranded DNA is then prepared from the pBCFNACAT plasmid containing the BPN' N76D/Y217L (the pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another oligonucleotide with the sequence

* * * * * *Pvu*II

- 5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3' (Seq. ID No.15)

- to obtain BPN' N76D/Q103A/Y104I/Y217L. All steps involved in the cloning, the single-stranded DNA preparation, the mutagenesis, and the screening for mutants are carried out as described above. Expression of the BPN' gene and its variants are achieved by integrating plasmid DNA (pBCFNACAT containing the different variants' BPN' gene) directly into a protease-deficient strain of *Bacillus subtilis* as described in RE 34,606.

- Numerous variants are made as per the teachings of these Protease Manufacture Examples. Kinetics data and stability data are generated for such variants. The kinetics data are generated using the methods described hereinbefore and are provided in Table V. The stability data are generated as detailed herein. Results are shown in Table VI.

Thermal Stability Assay Procedure

- Purified enzyme is buffer-exchanged into 0.1 M glycine pH 10.0, 0.01% Tween-80 by applying the enzyme to a column consisting of Sephadex G-25 equilibrated with this buffer and eluting the enzyme from the column using the same buffer.

To a tube containing 0.1 M glycine, 0.01% Tween-80 pH 10.0 thermostatted at 60°C, the buffer-exchanged enzyme is added to give a final enzyme concentration of 15 ug/ml.

- Aliquots are removed from the 60°C incubation at various times and immediately assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time.

Half-life, which is the length of time required for 50% enzyme inactivation, is determined from the first-order plot of reaction velocity as a function of the time of incubation at 60°C.

The data are presented in Table VI as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table V

| Enzyme | kcat (s ⁻¹) | KM (mM) | kcat/KM (s ⁻¹ M ⁻¹) |
|-----------------------------|----------------------------|------------|---|
| B. lentus subtilisin | 170 | 0.78 | 2.20E+05 |
| N76D/S103G/V104I* | 380 | 1.4 | 2.70E+05 |
| N76D/S103A/V104F | 730 | 0.33 | 2.20E+06 |
| N76D/S103A/V104N | 790 | 2.8 | 2.80E+05 |
| N76D/S103A/V104S | 170 | 0.83 | 2.00E+05 |
| N76D/S103A/V104T | 370 | 1.9 | 2.00E+05 |
| N76D/S103A/V104W | 880 | 0.31 | 2.80E+06 |
| N76D/S103A/V104Y | 690 | 0.5 | 1.40E+06 |
| K27R/N76D/V104Y/N123S | 500 | 1.2 | 4.20E+05 |
| N76D/S101G/S103A/V104I* | 620 | 1.3 | 4.80E+05 |
| N76D/S103A/V104I/S105A* | 550 | 1.3 | 4.20E+05 |
| N76D/S103A/V104I/S105D* | 440 | 1.7 | 2.60E+05 |
| N76D/S103A/V104T/I107A* | 120 | 5.7 | 2.10E+04 |
| N76D/S103A/V104T/I107L* | 310 | 3.2 | 9.70E+04 |
| N76D/S103A/V104I/L126A | 90 | 2.2 | 4.10E+04 |
| N76D/S103A/V104I/L126F | 180 | 1.9 | 9.50E+04 |
| N76D/S103A/V104I/L126I | 100 | 2.4 | 4.20E+04 |
| N76D/S103A/V104I/L126V | 64 | 3.2 | 2.00E+04 |
| N76D/S103A/V104I/S128G* | 560 | 1.7 | 3.30E+05 |
| N76D/S103A/V104I/S128L* | 430 | 3.8 | 1.10E+05 |
| N76D/S103A/V104I/L135A | 140 | 0.76 | 1.80E+05 |
| N76D/S103A/V104I/L135F | 390 | 0.69 | 5.70E+05 |
| N76D/S103A/V104I/L135I | 110 | 0.73 | 1.50E+05 |
| N76D/S103A/V104I/L135V | 140 | 0.86 | 1.60E+05 |
| N76D/S103A/V104I/S156E* | 170 | 2.6 | 6.50E+04 |
| N76D/S103A/V104I/S166D* | 160 | 3.5 | 4.60E+04 |
| N76D/S103A/V104I/D197E | 510 | 1.4 | 3.60E+05 |
| N76D/S103A/V104I/N204A* | 530 | 1.1 | 4.80E+05 |
| N76D/S103A/V104I/N204G* | 580 | 1.4 | 4.10E+05 |
| N76D/S103A/V104I/N204C* | 370 | 1.3 | 2.90E+05 |
| N76D/S103A/V104I/P210I* | 500 | 1.2 | 4.20E+05 |
| N76D/S103A/V104I/L217H* | 80 | 0.63 | 1.30E+05 |
| N76D/S103A/V104I/M222A | 70 | 3.1 | 2.30E+04 |
| N76D/S103A/V104I/M222S | 80 | 3.1 | 2.60E+04 |
| N76D/S103A/V104I/T260P | 660 | 1.5 | 4.40E+05 |
| N76D/S103A/V104I/S265N | 590 | 1.3 | 4.50E+05 |
| K27R/N76D/V104Y/I107V/N123S | 220 | 1.4 | 1.60E+05 |
| K27R/N76D/V104Y/N123S/D197E | 430 | 1.1 | 3.90E+05 |
| K27R/N76D/V104Y/N123S/N204C | 400 | 1.1 | 3.60E+05 |
| K27R/N76D/V104Y/N123S/Q206L | 440 | 1.2 | 3.70E+05 |

| | | | |
|---|-----|------|----------|
| K27R/N76D/V104Y/N123S/S216V | 440 | 1.2 | 3.70E+05 |
| K27R/N76D/V104Y/N123S/N218S | 760 | 0.98 | 7.80E+05 |
| K27R/N76D/V104Y/N123S/T260P | 410 | 1.2 | 3.40E+05 |
| K27R/N76D/V104Y/N123S/T274A | 390 | 1 | 3.90E+05 |
| N76D/S103A/V104I/L126F/S265N | 170 | 2.1 | 8.10E+04 |
| N76D/S103A/V104I/S156E/S166D* | 40 | 6.3 | 6.40E+03 |
| K27R/N76D/V104Y/N123S/G195E/G197E | 410 | 0.98 | 4.20E+05 |
| K27R/N76D/V104Y/N123S/G195E/N218S | 540 | 0.66 | 8.20E+05 |
| K27R/N76D/V104Y/N123S/D197E/N218S | 770 | 0.79 | 9.80E+05 |
| K27R/N76D/V104Y/N123S/N204C/N218S | 610 | 0.99 | 6.20E+05 |
| K27R/N76D/V104Y/N123S/Q206L/N218S | 580 | 0.78 | 7.40E+05 |
| K27R/N76D/V104Y/N123S/N218S/T260P | 660 | 1 | 6.60E+05 |
| K27R/N76D/V104Y/N123S/N218S/T274A | 590 | 0.89 | 6.60E+05 |
| K27R/N76D/V104Y/Q109S/N123S/N218S/T274A | 520 | 1 | 5.20E+05 |
| K27R/N76D/V104Y/N123S/G195E/D197E/N218S | 460 | 0.65 | 7.10E+05 |
| B. amyloliquefaciens subtilisin (BPN') | 50 | 0.14 | 3.60E+05 |
| BPN'-N76D/Y217L* | 380 | 0.46 | 8.30E+05 |

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

5

Table VI

| Enzyme | Thermal Stability (% half-life of native enzyme) |
|----------------------|---|
| B. lentus subtilisin | 100 |
| N76D | 590 |
| N76D/S99D | 840 |
| N76D/S103A | 390 |
| N76D/V104I | 660 |
| N76D/I107V | 710 |
| N76D/N123S | 70 |
| N76D/S99D/S101R | 610 |
| N76D/S99D/S103A | 590 |
| N76D/S99D/V104I | 910 |
| N76D/S101R/S103A | 930 |
| N76D/S101R/V104I | 500 |
| N76D/S103A/V104I | 460 |
| N76D/S103G/V104I* | 370 |
| N76D/S103A/V104F | 480 |
| N76D/S103A/V104N | 230 |
| N76D/S103A/V104S | 230 |
| N76D/S103A/V104T | 370 |

| | |
|------------------------------|------|
| N76D/S103A/V104W | 280 |
| N76D/S103A/V104Y | 400 |
| N76D/V104I/I107V | 940 |
| N76D/V104Y/I107V | 820 |
| N76D/V104I/N123S | 80 |
| N76D/I107V/N123S | 150 |
| K27R/N76D/V104Y/N123S | 100 |
| N76D/S99D/S101R/S103A | 570 |
| N76D/S99D/S101R/V104I | 1000 |
| N76D/S99D/S103A/V104I | 680 |
| N76D/S101G/S103A/V104I* | 390 |
| N76D/S101R/S103A/V104I | 470 |
| N76D/S103A/V104I/S105A* | 360 |
| N76D/S103A/V104I/S105D* | 370 |
| N76D/S103A/V104T/I107A* | 270 |
| N76D/S103A/V104T/I107L* | 230 |
| N76D/S103A/V104I/N123S | 110 |
| N76D/V104I/I107V/N123S | 220 |
| N76D/S103A/V104I/L126A | 270 |
| N76D/S103A/V104I/L126F | 950 |
| N76D/S103A/V104I/L126I | 410 |
| N76D/S103A/V104I/L126V | 320 |
| N76D/S103A/V104I/S128G* | 640 |
| N76D/S103A/V104I/S128L* | 760 |
| N76D/S103A/V104I/L135A | 230 |
| N76D/S103A/V104I/L135F | 200 |
| N76D/S103A/V104I/L135I | 510 |
| N76D/S103A/V104I/L135V | 500 |
| N76D/S103A/V104I/S156E* | 120 |
| N76D/S103A/V104I/S166D* | 590 |
| N76D/S103A/V104I/D197E | 460 |
| N76D/S103A/V104I/N204A* | 230 |
| N76D/S103A/V104I/N204G* | 240 |
| N76D/S103A/V104I/N204C* | 500 |
| N76D/S103A/V104I/P210I* | 1370 |
| N76D/S103A/V104I/L217H* | 60 |
| N76D/S103A/V104I/M222A | 520 |
| N76D/S103A/V104I/M222S | 490 |
| N76D/S103A/V104I/T260P | 490 |
| N76D/S103A/V104I/S265N | 360 |
| K27R/N76D/V104Y/I107V/N123S | 210 |
| K27R/N76D/V104Y/N123S/D197E | 120 |
| K27R/N76D/V104Y/N123S/N204C | 110 |
| K27R/N76D/V104Y/N123S/Q206L | 380 |
| K27R/N76D/V104Y/N123S/S216V | 140 |
| K27R/N76D/V104Y/N123S/N218S | 270 |
| K27R/N76D/V104Y/N123S/T260P | 40 |
| K27R/N76D/V104Y/N123S/T274A | 60 |
| N76D/S99D/S101R/S103A/V104I | 590 |
| N76D/S99D/S103A/V104I/N123S | 110 |
| N76D/S103A/V104I/L126F/S265N | 810 |

| | |
|--|---------|
| N76D/S103A/V104I/S156E/S166D* | 220 |
| K27R/N76D/V104Y/N123S/G195E/G197E | 90 |
| K27R/N76D/V104Y/N123S/G195E/N218S | 250 |
| K27R/N76D/V104Y/N123S/D197E/N218S | 270 |
| K27R/N76D/V104Y/N123S/N204C/N218S | 460 |
| K27R/N76D/V104Y/N123S/Q206L/N218S | 1400 |
| K27R/N76D/V104Y/N123S/N218S/T260P | 310 |
| * K27R/N76D/V104Y/N123S/N218S/T274A | 180 |
| N76D/S99D/S101R/S103A/V104I/N123S | 90 |
| K27R/N76D/V104Y/Q109S/N123S/N218S/T274 | 230 |
| K27R/N76D/V104Y/N123S/G195E/D197E/N21 | 240 |
| B. amyloliquefaciens subtilisin (BPN') | 100 |
| BPN'-N76D/Y217L* | 420 |

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

2. Cleaning Composition Materials:

5 The cleaning compositions of the present invention also comprise, in addition to the protease enzyme described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. The term "cleaning composition materials", as used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition
10 desired and the form of the product (e.g., liquid; granule; spray composition), which materials are also compatible with the protease enzyme used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g.,
15 through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the protease enzyme to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

20 An effective amount of one or more protease enzymes described above are included in compositions useful for cleaning a variety of surfaces in need of proteinaceous stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for cleaning fabrics, unlimited in form (e.g.,
25 granular, liquid and bar formulations); dishwashing compositions (unlimited in form); oral cleaning compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); and denture cleaning compositions, unlimited in

form (e.g., liquid, tablet). As used herein, "effective amount of protease enzyme" refers to the quantity of protease enzyme described herein before necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of
5 ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like.

Preferably the cleaning compositions of the present invention comprise
10 from about 0.0001% to about 10% of one or more protease enzymes, more preferably from about 0.001% to about 1%, more preferably still from about 0.001% to about 0.1%. Several examples of various cleaning compositions wherein the protease enzymes may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless
15 otherwise specified.

As used herein, "non-fabric cleaning compositions" include hard surface cleaning compositions, dishwashing compositions, oral cleaning compositions, denture cleaning compositions and personal cleansing compositions.

A. Cleaning Compositions for Hard Surfaces, Dishes and Fabrics

20 The protease enzymes can be used in any detergent composition where high sudsing and/or good insoluble substrate removal are desired. Thus the protease enzymes can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions and the like. Such compositions can be in the
25 form of liquids, granules, bars and the like. Such compositions can be formulated as modern "concentrated" detergents which contain as much as 30%-60% by weight of surfactants.

The cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are
30 typically present at levels of from about 0.1% to about 60%, preferably from about 1% to about 35%, of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C₁₁-C₁₈ alkyl benzene sulfonates and primary and random alkyl sulfates, the C₁₀-C₁₈ secondary (2,3) alkyl sulfates of the formulas
35 $\text{CH}_3(\text{CH}_2)_x(\text{CHOSO}_3^- \text{M}^+)\text{CH}_3$ and $\text{CH}_3(\text{CH}_2)_y(\text{CHOSO}_3^- \text{M}^+)\text{CH}_2\text{CH}_3$ wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C₁₀-C₁₈ alkyl

alkoxy sulfates (especially EO 1-7 ethoxy sulfates), C₁₀-C₁₈ alkyl alkoxy carboxylates (especially the EO 1-7 ethoxycarboxylates), the C₁₀-C₁₈ alkyl polyglycosides, and their corresponding sulfated polyglycosides, C₁₂-C₁₈ alpha-sulfonated fatty acid esters, C₁₂-C₁₈ alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C₁₂-C₁₈ betaines and sulfobetaines ("sultaines"), C₁₀-C₁₈ amine oxides, C₈-C₂₄ sarcosinates (especially oleoyl sarcosinate), and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C₁₀-C₁₈ N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

Particularly useful is the class of nonionic surfactants which are condensates of ethylene oxide with a hydrophobic moiety to provide a surfactant having an average hydrophilic-lipophilic balance (HLB) in the range from 5 to 17, preferably from 6 to 14, more preferably from 7 to 12. The hydrophobic (lipophilic) moiety may be aliphatic or aromatic in nature and the length of the polyoxyethylene group which is condensed with any particular hydrophobic group can be readily adjusted to yield a water-soluble compound having the desired degree of balance between hydrophilic and hydrophobic elements. Especially preferred are the C₉-C₁₅ primary alcohol ethoxylates (or mixed ethoxy/propoxy) containing 3-8 moles of ethylene oxide per mole of alcohol, particularly the C₁₄-C₁₅ primary alcohols containing 6-8 moles of ethylene oxide per mole of alcohol, the C₁₂-C₁₅ primary alcohols containing 3-5 moles of ethylene oxide per mole of alcohol, and mixtures thereof.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C₁₀-C₁₆ alkalamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C₁₀-C₁₄ monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as MgCl₂, MgSO₄,

and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases, peroxidases, and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various detergent and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Various soil release agents, especially of the anionic oligoester type, various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially thoxylated tetraethylene pentamine, various dispersing agents, especially

polyacrylates and polyasparatates, various brighteners, especially anionic brighteners, various dye transfer inhibiting agents, such as polyvinyl pyrrolidone, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays and clay flocculating polymers (e.g., poly(oxy ethylene)), and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions of the present invention. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

1. Hard surface cleaning compositions

As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 5%, more preferably still from about 0.001% to about 1% by weight of active protease enzyme of the composition. In addition to comprising one or more protease enzymes, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy/streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%. Preferably the pH should be in the range of about 8 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether,

diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as isopropanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

The hard surface cleaning composition embodiment of the present invention is illustrated by the following nonlimiting examples. (In the following examples, reference to "Protease #" in the examples is to the variant useful in the present invention compositions having the given number in Table III hereinbefore.)

Examples 1-6
Liquid Hard Surface Cleaning Compositions

| | | Example No. | | | | | |
|----|---|-----------------|------|------|------|------|------|
| 20 | Component | 1 | 2 | 3 | 4 | 5 | 6 |
| | Protease # 12 | 0.05 | 0.20 | 0.02 | 0.03 | 0.10 | 0.03 |
| | Protease # 4 | — | — | — | — | 0.20 | 0.02 |
| | EDTA** | — | — | 2.90 | 2.90 | — | — |
| | Na Citrate | — | — | — | — | 2.90 | 2.90 |
| 25 | NaC ₁₂ Alkyl-benzene sulfonate | 1.95 | — | 1.95 | — | 1.95 | — |
| | NaC ₁₂ Alkylsulfate | — | 2.20 | — | 2.20 | — | 2.20 |
| | NaC ₁₂ (ethoxy)*** sulfate | — | 2.20 | — | 2.20 | — | 2.20 |
| 30 | C ₁₂ Dimethylamine oxide | — | 0.50 | — | 0.50 | — | 0.50 |
| | Na Cumene sulfonate | 1.30 | — | 1.30 | — | 1.30 | — |
| | Hexyl Carbitol*** | 6.30 | 6.30 | 6.30 | 6.30 | 6.30 | 6.30 |
| | Water**** | balance to 100% | | | | | |

35 **Na₄ thylenediamine diacetic acid

***Diethyleneglycol monohexyl ether

****All formulas adjusted to pH 7

In Examples 1-4 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

5 In Examples 5 and 6, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 7-12

10 Spray Compositions for Cleaning Hard Surfaces and Removing Household Mildew

| | | Example No. | | | | | |
|-----------|------------------------|-----------------|------|------|------|------|------|
| Component | | 7 | 8 | 9 | 10 | 11 | 12 |
| | Protease # 12 | 0.20 | 0.05 | 0.10 | 0.30 | 0.20 | 0.30 |
| 15 | Protease # 4 | - | - | - | - | 0.30 | 0.10 |
| | Sodium octyl sulfate | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| | Sodium dodecyl sulfate | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| | Sodium hydroxide | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 |
| | Silicate (Na) | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |
| 20 | Perfume | 0.35 | 0.35 | 0.35 | 0.35 | 0.35 | 0.35 |
| | Water | balance to 100% | | | | | |

Product pH is about 7.

In Examples 7-10 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 11 and 12, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

2. Dishwashing Compositions

In another embodiment of the present invention, dishwashing compositions comprise one or more protease enzymes. As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to, granular and liquid forms. The dishwashing composition embodiment of the present invention is illustrated by the following examples.

Examples 13-18
Dishwashing Composition

| | | Example No. | | | | | |
|-----------|---|-----------------|-------|-------|-------|-------|-------|
| Component | | 13 | 14 | 15 | 16 | 17 | 18 |
| 5 | Protease # 12 | 0.05 | 0.50 | 0.02 | 0.40 | 0.10 | 0.03 |
| | Protease # 4 | - | - | - | - | 0.40 | 0.02 |
| | C ₁₂ -C ₁₄ N-methyl-glucamide | 0.90 | 0.90 | 0.90 | 0.90 | 0.90 | 0.90 |
| | C ₁₂ ethoxy (1) sulfate | 12.00 | 12.00 | 12.00 | 12.00 | 12.00 | 12.00 |
| 10 | 2-methyl undecanoic acid | 4.50 | 4.50 | - | 4.50 | 4.50 | - |
| | C ₁₂ ethoxy (2) carboxylate | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 | 4.50 |
| | C ₁₂ alcohol ethoxylate (4) | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| | C ₁₂ amine oxide | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| | Sodium cumene sulfonate | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| 15 | Ethanol | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| | Mg ⁺⁺ (as MgCl ₂) | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| | Ca ⁺⁺ (as CaCl ₂) | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| | Water | balance to 100% | | | | | |

Product pH is adjusted to 7.

20 In Examples 13-16 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

25 In Examples 17 and 18, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4 with substantially similar results.

Example 19Granular Automatic Dishwashing Composition

| <u>Component</u> | <u>A</u> | <u>B</u> | <u>C</u> |
|---|-----------------|----------|----------|
| Citric Acid | 15.0 | - | - |
| Citrate | 4.0 | 29.0 | 15.0 |
| Acrylate/methacrylate copolymer | 6.0 | - | 6.0 |
| Acrylic acid maleic acid copolymer | - | 3.7 | - |
| Dry add carbonate | 9.0 | - | 20.0 |
| Alkali metal silicate | 8.5 | 17.0 | 9.0 |
| Paraffin | - | 0.5 | - |
| Benzotriazole | - | 0.3 | - |
| Termamyl 60T | 1.5 | 1.5 | 1.0 |
| Protease # 12 (4.6% prill) | 1.6 | 1.6 | 1.6 |
| Percarbonate (AvO) | 1.5 | - | - |
| Perborate monohydrate | - | 0.3 | 1.5 |
| Perborate tetrahydrate | - | 0.9 | - |
| Tetraacetylene diamine | 3.8 | 4.4 | - |
| Diethylene triamine penta methyl phosphonic acid (Mg salt) | 0.13 | 0.13 | 0.13 |
| Alkyl ethoxy sulphate - 3 times ethoxylated | 3.0 | - | - |
| Alkyl ethoxy propoxy nonionic surfactant | - | 1.5 | - |
| Suds suppressor | 2.0 | - | - |
| Olin SLF18 nonionic surfactant | - | - | 2.0 |
| Sulphate | Balance to 100% | | |

- 5 In Examples 19 A-C the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in Examples 19 A-C, any combination of the proteases useful in the present invention recited in Tables III, V and VI among
- 10 others, are substituted for Protease # 12 with substantially similar results.

3. Fabric cleaning compositions

- In another embodiment of the present invention, fabric cleaning compositions comprise one or more protease enzymes. As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for
- 15 cleaning fabrics, including but not limited to, granular, liquid and bar forms.

a. Granular fabric cleaning compositions

The granular fabric cleaning compositions of the present invention contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%,
 5 more preferably from about 0.01% to about 1% by weight of active protease enzyme of the composition. In addition to one or more protease enzymes, the granular fabric cleaning compositions typically comprise at least one surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present
 10 invention is illustrated by the following examples.

Examples 20-23

Granular Fabric Cleaning Composition

| | | Example No. | | | |
|------------------|--|-----------------|-----------|-----------|-----------|
| <u>Component</u> | | <u>20</u> | <u>21</u> | <u>22</u> | <u>23</u> |
| 15 | Protease # 12 (4% Prill) | 0.10 | 0.20 | 0.03 | 0.05 |
| | Protease # 4 (4% Prill) | - | - | 0.02 | 0.05 |
| | C ₁₃ linear alkyl benzene sulfonate | 22.00 | 22.00 | 22.00 | 22.00 |
| | Phosphate (as sodium tripolyphosphates) | 23.00 | 23.00 | 23.00 | 23.00 |
| 20 | Sodium carbonate | 23.00 | 23.00 | 23.00 | 23.00 |
| | Sodium silicate | 14.00 | 14.00 | 14.00 | 14.00 |
| | Zeolite | 8.20 | 8.20 | 8.20 | 8.20 |
| | Chelant (diethylenetriamine-pentaacetic acid) | 0.40 | 0.40 | 0.40 | 0.40 |
| 25 | Sodium sulfate | 5.50 | 5.50 | 5.50 | 5.50 |
| | Water | balance to 100% | | | |

In Examples 20-21 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with
 30 substantially similar results.

In Examples 22 and 23, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 24-27
Granular Fabric Cleaning Composition

| | | Example No. | | | |
|----|---|------------------------|-------|-------|-------|
| | Component | 24 | 25 | 26 | 27 |
| 5 | Protease # 12 (4% Prill) | 0.10 | 0.20 | 0.03 | 0.05 |
| 6 | Protease # 4 (4% Prill) | - | - | 0.02 | 0.05 |
| | C ₁₂ alkyl benzene sulfonate | 12.00 | 12.00 | 12.00 | 12.00 |
| | Zeolite A (1-10 micrometer) | 26.00 | 26.00 | 26.00 | 26.00 |
| | 2-butyl octanoic acid | 4.00 | 4.00 | 4.00 | 4.00 |
| 10 | C ₁₂ -C ₁₄ secondary (2,3) alkyl sulfate, Na salt | 5.00 | 5.00 | 5.00 | 5.00 |
| | Sodium citrate | 5.00 | 5.00 | 5.00 | 5.00 |
| | Optical brightener | 0.10 | 0.10 | 0.10 | 0.10 |
| | Sodium sulfate | 17.00 | 17.00 | 17.00 | 17.00 |
| 15 | <u>Fillers, water, minors</u> | <u>balance to 100%</u> | | | |

In Examples 24 and 25 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

- 20 In Examples 26 and 27, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 28 and 29Granular Fabric Cleaning Compositions

| <u>Components</u> | <u>Example No.</u> | |
|---|--------------------|-----------|
| | <u>28</u> | <u>29</u> |
| Linear alkyl benzene sulphonate | 11.4 | 10.70 |
| Tallow alkyl sulphate | 1.80 | 2.40 |
| C ₁₄₋₁₅ alkyl sulphate | 3.00 | 3.10 |
| C ₁₄₋₁₅ alcohol 7 times ethoxylated | 4.00 | 4.00 |
| Tallow alcohol 11 times ethoxylated | 1.80 | 1.80 |
| Dispersant | 0.07 | 0.1 |
| Silicone fluid | 0.80 | 0.80 |
| Trisodium citrate | 14.00 | 15.00 |
| Citric acid | 3.00 | 2.50 |
| Zeolite | 32.50 | 32.10 |
| Maleic acid acrylic acid copolymer | 5.00 | 5.00 |
| Diethylene triamine penta methylene phosphonic acid | 1.00 | 0.20 |
| Protease # 12 (4% Prill) | 0.30 | 0.30 |
| Lipase | 0.36 | 0.40 |
| Amylase | 0.30 | 0.30 |
| Sodium silicate | 2.00 | 2.50 |
| Sodium sulphate | 3.50 | 5.20 |
| Polyvinyl pyrrolidone | 0.30 | 0.50 |
| Perborate | 0.5 | 1 |
| Phenol sulphonate | 0.1 | 0.2 |
| Peroxidase | 0.1 | 0.1 |
| Minors | Up to 100 | Up to 100 |

5

Examples 30 and 31Granular Fabric Cleaning Compositions

| <u>Components</u> | <u>Example No.</u> | |
|---|--------------------|-----------|
| | <u>30</u> | <u>31</u> |
| Sodium linear C ₁₂ alkyl benzene-sulfonate | 6.5 | 8.0 |
| Sodium sulfate | 15.0 | 18.0 |
| Zeolit A | 26.0 | 22.0 |
| Sodium nitrilotriacetat | 5.0 | 5.0 |
| Polyvinyl pyrrolidone | 0.5 | 0.7 |
| Tetraacetylene diamine | 3.0 | 3.0 |

| | | |
|--|-----------|-----------|
| Boric acid | 4.0 | - |
| Perborate | 0.5 | 1 |
| Phenol sulphonate | 0.1 | 0.2 |
| Protease # 12 (4% Prill) | 0.4 | 0.4 |
| Fillers (e.g., silicates; carbonates; perfumes; water) | Up to 100 | Up to 100 |

Example 32Compact Granular Fabric Cleaning Composition

| <u>Components</u> | <u>Weight %</u> |
|--|-----------------|
| Alkyl Sulphate | 8.0 |
| Alkyl Ethoxy Sulphate | 2.0 |
| Mixture of C25 and C45 alcohol 3 and 7 times ethoxylated | 6.0 |
| Polyhydroxy fatty acid amide | 2.5 |
| Zeolite | 17.0 |
| Layered silicate/citrate | 16.0 |
| Carbonate | 7.0 |
| Maleic acid acrylic acid copolymer | 5.0 |
| Soil release polymer | 0.4 |
| Carboxymethyl cellulose | 0.4 |
| Poly (4-vinylpyridine) -N-oxide | 0.1 |
| Copolymer of vinylimidazole and vinylpyrrolidone | 0.1 |
| PEG2000 | 0.2 |
| Protease # 12 (4% Prill) | 0.5 |
| Lipase | 0.2 |
| Cellulase | 0.2 |
| Tetracetylene diamine | 6.0 |
| Percarbonate | 22.0 |
| Ethylene diamine disuccinic acid | 0.3 |
| Suds suppressor | 3.5 |
| Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate | 0.25 |
| Disodium-4,4'-bis (2-sulfostyryl) biphenyl | 0.05 |
| Water, Perfume and Minors | Up to 100 |

Example 33Granular Fabric Cleaning Composition

| | | |
|---|--------------------------------|-----------------|
| 5 | <u>Component</u> | <u>Weight %</u> |
| | Linear alkyl benzen sulphonate | 7.6 |

| | |
|---|-----------|
| C ₁₆ -C ₁₈ alkyl sulfate | 1.3 |
| C ₁₄ - ₁₅ alcohol 7 times ethoxylated | 4.0 |
| Coco-alkyl-dimethyl hydroxyethyl ammonium chloride | 1.4 |
| Dispersant | 0.07 |
| Silicone fluid | 0.8 |
| € Trisodium citrate | 5.0 |
| Zeolite 4A | 15.0 |
| Maleic acid acrylic acid copolymer | 4.0 |
| Diethylene triamine penta methylene phosphonic acid | 0.4 |
| Perborate | 15.0 |
| Tetraacetylene diamine | 5.0 |
| Smectite clay | 10.0 |
| Poly (oxy ethylene) (MW 300,000) | 0.3 |
| Protease # 12 (4% Prill) | 0.4 |
| Lipase | 0.2 |
| Amylase | 0.3 |
| Cellulase | 0.2 |
| Sodium silicate | 3.0 |
| Sodium carbonate | 10.0 |
| Carboxymethyl cellulose | 0.2 |
| Brighteners | 0.2 |
| Water, perfume and minors | Up to 100 |

Example 34Granular Fabric Cleaning Composition

| <u>Component</u> | <u>Weight %</u> |
|--|-----------------|
| Linear alkyl benzene sulfonate | 6.92 |
| Tallow alkyl sulfate | 2.05 |
| C ₁₄ - ₁₅ alcohol 7 times ethoxylated | 4.4 |
| C ₁₂ - ₁₅ alkyl ethoxy sulfate - 3 times ethoxylated | 0.16 |
| Zeolite | 20.2 |
| Citrate | 5.5 |
| Carbonate | 15.4 |
| Silicate | 3.0 |
| Maleic acid acrylic acid copolymer | 4.0 |
| Carboxymethyl cellulase | 0.31 |
| Soil release polymer | 0.30 |
| Prot as # 12 (4% Prill) | 0.2 |

| | |
|---|-----------|
| Lipase | 0.36 |
| Cellulase | 0.13 |
| Perborate tetrahydrate | 11.64 |
| Perborate monohydrate | 8.7 |
| Tetraacetylene diamine | 5.0 |
| Diethylene tramine penta methyl phosphonic acid | 0.38 |
| Magnesium sulfate | 0.40 |
| Brightener | 0.19 |
| Perfume, silicone, suds suppressors | 0.85 |
| Minors | Up to 100 |

In each of Examples 28-34 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 28-34, any combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

b. Liquid fabric cleaning compositions

Liquid fabric cleaning compositions of the present invention comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.001% to about 0.1%, by weight of active protease enzyme of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a fatty acid, a water-soluble detergency builder and water.

The liquid fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 35-39
Liquid Fabric Cleaning Compositions

| | | Example No. | | | | |
|----|---|------------------------|-------|-------|-------|-------|
| | Component | 35 | 36 | 37 | 38 | 39 |
| 5 | Protease # 12 | 0.05 | 0.03 | 0.30 | 0.03 | 0.10 |
| | Protease # 4 | — | — | — | 0.01 | 0.20 |
| | C ₁₂ - C ₁₄ alkyl sulfate, Na | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| | 2-Butyl octanoic acid | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| | Sodium citrate | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 10 | C ₁₀ alcohol ethoxylate (3) | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| | Monethanolamine | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 |
| | <u>Water/propylene glycol/ethanol (100:1:1)</u> | <u>balance to 100%</u> | | | | |

In Examples 35-37 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 38 and 39, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 40-41Liquid Fabric Cleaning Compositions

| | | <u>Example No.</u> | |
|------------------|--|--------------------|-----------|
| <u>Component</u> | | <u>40</u> | <u>41</u> |
| 5 | C ₁₂₋₁₄ alkenyl succinic acid | 3.0 | 8.0 |
| | Citric acid monohydrate | 10.0 | 15.0 |
| | Sodium C ₁₂₋₁₅ alkyl sulphate | 8.0 | 8.0 |
| | Sodium sulfate of C ₁₂₋₁₅ alcohol 2 times ethoxylated | - | 3.0 |
| | C ₁₂₋₁₅ alcohol 7 times ethoxylated | - | 8.0 |
| 10 | C ₁₂₋₁₅ alcohol 5 times ethoxylated | 8.0 | - |
| | Diethylene triamine penta (methylene phosphonic acid) | 0.2 | - |
| | Oleic acid | 1.8 | - |
| | Ethanol | 4.0 | 4.0 |
| | Propanediol | 2.0 | 2.0 |
| 15 | Protease # 12 | 0.2 | 0.2 |
| | Polyvinyl pyrrolidone | 1.0 | 2.0 |
| | Suds suppressor | 0.15 | 0.15 |
| | NaOH | up to pH 7.5 | |
| | Perborate | 0.5 | 1 |
| 20 | Phenol sulphonate | 0.1 | 0.2 |
| | Peroxidase | 0.4 | 0.1 |
| | Waters and minors | up to 100 parts | |

In each of Examples 40 and 41 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 40 and 41, any combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted from Protease # 12 with substantially similar results.

30 c. Bar fabric cleaning compositions

Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

35 The bar fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 42-45
Bar Fabric Cleaning Compositions

| | | Example No. | | | |
|-----------|--|-----------------|------|------|-------|
| Component | | 42 | 43 | 44 | 45 |
| 5 | Protease # 12 | 0.3 | - | 0.1 | 0.02 |
| | Protease # 4 | - | - | 0.4 | 0.03 |
| | C ₁₂ -C ₁₆ alkyl sulfate, Na | 20.0 | 20.0 | 20.0 | 20.00 |
| | C ₁₂ -C ₁₄ N-methyl glucamide | 5.0 | 5.0 | 5.0 | 5.00 |
| | C ₁₁ -C ₁₃ alkyl benzene sulfonate, Na | 10.0 | 10.0 | 10.0 | 10.00 |
| 10 | Sodium carbonate | 25.0 | 25.0 | 25.0 | 25.00 |
| | Sodium pyrophosphate | 7.0 | 7.0 | 7.0 | 7.00 |
| | Sodium tripolyphosphate | 7.0 | 7.0 | 7.0 | 7.00 |
| | Zeolite A (0.1-.10 μ) | 5.0 | 5.0 | 5.0 | 5.00 |
| | Carboxymethylcellulose | 0.2 | 0.2 | 0.2 | 0.20 |
| | Polyacrylate (MW 1400) | 0.2 | 0.2 | 0.2 | 0.20 |
| 15 | Coconut monethanolamide | 5.0 | 5.0 | 5.0 | 5.00 |
| | Brightener, perfume | 0.2 | 0.2 | 0.2 | 0.20 |
| | CaSO ₄ | 1.0 | 1.0 | 1.0 | 1.00 |
| | MgSO ₄ | 1.0 | 1.0 | 1.0 | 1.00 |
| | Water | 4.0 | 4.0 | 4.0 | 4.00 |
| 20 | Filler* | balance to 100% | | | |

*Can be selected from convenient materials such as CaCO₃, talc, clay, silicates, and the like.

In Examples 42 and 43 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 44 and 45, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

B. Additional Cleaning Compositions

In addition to the hard surface cleaning, dishwashing and fabric cleaning compositions discussed above, one or more protease enzymes may be incorporated into a variety of other cleaning compositions where hydrolysis of an insoluble substrate is desired. Such additional cleaning compositions

include but are not limited to, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning compositions.

1. Oral cleaning compositions

In another embodiment of the present invention, a pharmaceutically-
5 acceptable amount of one or more protease enzymes are included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment
10 solutions, and the like. Preferably, oral cleaning compositions of the present invention comprise from about 0.0001% to about 20% of one or more protease enzymes, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-
15 acceptable" means that drugs, medicaments or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier
20 components of the oral cleaning compositions of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The pharmaceutically-acceptable carrier components and optional
25 components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in U.S. Patent 5,096,700, Seibel, issued March 17, 1992; U.S. Patent 5,028,414, Sampathkumar, issued July 2, 1991; and U.S. Patent 5,028,415, Benedict, Bush and Sunberg, issued July 2,
30 1991; all of which are incorporated herein by reference.

The oral cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 46-49
Dentifrice Composition

| | | Example No. | | | |
|----|---|-----------------|--------|--------|--------|
| | Component | 46 | 47 | 48 | 49 |
| 5 | Protease # 12 | 2.000 | 3.500 | 1.500 | 2.000 |
| | Sorbitol (70% aqueous solution) | 35.000 | 35.000 | 35.000 | 35.000 |
| | PEG-6* | 1.000 | 1.000 | 1.000 | 1.000 |
| | Silica dental abrasive** | 20.000 | 20.000 | 20.000 | 20.000 |
| | Sodium fluoride | 0.243 | 0.243 | 0.243 | 0.243 |
| 10 | Titanium dioxide | 0.500 | 0.500 | 0.500 | 0.500 |
| | Sodium saccharin | 0.286 | 0.286 | 0.286 | 0.286 |
| | Sodium alkyl sulfate (27.9% aqueous solution) | 4.000 | 4.000 | 4.000 | 4.000 |
| | Flavor | 1.040 | 1.040 | 1.040 | 1.040 |
| 15 | Carboxyvinyl Polymer*** | 0.300 | 0.300 | 0.300 | 0.300 |
| | Carrageenan**** | 0.800 | 0.800 | 0.800 | 0.800 |
| | Water | balance to 100% | | | |

*PEG-6 = Polyethylene glycol having a molecular weight of 600.

**Precipitated silica identified as Zeodent 119 offered by J.M. Huber.

20 ***Carbopol offered by B.F. Goodrich Chemical Company.

****Iota Carrageenan offered by Hercules Chemical Company.

25 In Examples 46-49 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 46-49, any combination of the protease enzymes useful in the present invention recited in Tables III, V, VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 50-53
Mouthwash Composition

| | | Example No. | | | |
|----|------------------|-----------------|-------|-------|-------|
| | Component | 50 | 51 | 52 | 53 |
| 5 | Protease # 12 | 3.00 | 7.50 | 1.00 | 5.00 |
| | SDA 40 Alcohol | 8.00 | 8.00 | 8.00 | 8.00 |
| | Flavor | 0.08 | 0.08 | 0.08 | 0.08 |
| | Emulsifier | 0.08 | 0.08 | 0.08 | 0.08 |
| | Sodium Fluoride | 0.05 | 0.05 | 0.05 | 0.05 |
| 10 | Glycerin | 10.00 | 10.00 | 10.00 | 10.00 |
| | Sweetener | 0.02 | 0.02 | 0.02 | 0.02 |
| | Benzoic acid | 0.05 | 0.05 | 0.05 | 0.05 |
| | Sodium hydroxide | 0.20 | 0.20 | 0.20 | 0.20 |
| | Dye | 0.04 | 0.04 | 0.04 | 0.04 |
| 15 | Water | balance to 100% | | | |

In Examples 50-53 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 50-53, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 54-57
Lozenge Composition

| | | Example No. | | | |
|----|---------------|-----------------|-------|-------|-------|
| | Component | 54 | 55 | 56 | 57 |
| | Protease # 12 | 0.01 | 0.03 | 0.10 | 0.02 |
| | Sorbitol | 17.50 | 17.50 | 17.50 | 17.50 |
| | Mannitol | 17.50 | 17.50 | 17.50 | 17.50 |
| 30 | Starch | 13.60 | 13.60 | 13.60 | 13.60 |
| | Sweetener | 1.20 | 1.20 | 1.20 | 1.20 |
| | Flavor | 11.70 | 11.70 | 11.70 | 11.70 |
| | Color | 0.10 | 0.10 | 0.10 | 0.10 |
| | Corn Syrup | balance to 100% | | | |

In Examples 54-57 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with

substantially similar results. Also in Examples 54-57, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

5

Examples 58-61
Chewing Gum Composition

| | | Example No. | | | |
|-----------|---------------------------------|-------------|-------|-------|-------|
| Component | | 58 | 59 | 60 | 61 |
| | Protease # 12 | 0.03 | 0.02 | 0.10 | 0.05 |
| 10 | Sorbitol crystals | 38.44 | 38.40 | 38.40 | 38.40 |
| | Paloja-T gum base* | 20.00 | 20.00 | 20.00 | 20.00 |
| | Sorbitol (70% aqueous solution) | 22.00 | 22.00 | 22.00 | 22.00 |
| | Mannitol | 10.00 | 10.00 | 10.00 | 10.00 |
| | Glycerine | 7.56 | 7.56 | 7.56 | 7.56 |
| 15 | Flavor | 1.00 | 1.00 | 1.00 | 1.00 |

*Supplied by L.A. Dreyfus Company.

In Examples 58-61 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 58-61, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

2. Denture cleaning compositions

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more protease enzymes. Such denture cleaning compositions comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 50% of one or more protease enzymes, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see for example U.S. Patent 5,055,305, Young, incorporated herein by reference), and are generally appropriate for incorporation of one or more protease enzymes for removing proteinaceous stains from dentures.

The denture cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 62-65

Two-layer Effervescent Denture Cleansing Tablet

| 5 | Component | Example No. | | | |
|----|------------------------------|-------------|------|-------|-------|
| | | 62 | 63 | 64 | 65 |
| | <u>Acidic Layer</u> | | | | |
| | Protease # 12 | 1.0 | 1.5 | 0.01 | 0.05 |
| | Tartaric acid | 24.0 | 24.0 | 24.00 | 24.00 |
| 10 | Sodium carbonate | 4.0 | 4.0 | 4.00 | 4.00 |
| | Sulphamic acid | 10.0 | 10.0 | 10.00 | 10.00 |
| | PEG 20,000 | 4.0 | 4.0 | 4.00 | 4.00 |
| | Sodium bicarbonate | 24.5 | 24.5 | 24.50 | 24.50 |
| | Potassium persulfate | 15.0 | 15.0 | 15.00 | 15.00 |
| 15 | Sodium acid pyrophosphate | 7.0 | 7.0 | 7.00 | 7.00 |
| | Pyrogenic silica | 2.0 | 2.0 | 2.00 | 2.00 |
| | Tetracetylene diamine | 7.0 | 7.0 | 7.00 | 7.00 |
| | Ricinoleylsulfosuccinate | 0.5 | 0.5 | 0.50 | 0.50 |
| | Flavor | 1.0 | 1.0 | 1.00 | 1.00 |
| 20 | <u>Alkaline Layer</u> | | | | |
| | Sodium perborate monohydrate | 32.0 | 32.0 | 32.00 | 32.00 |
| | Sodium bicarbonate | 19.0 | 19.0 | 19.00 | 19.00 |
| | EDTA | 3.0 | 3.0 | 3.00 | 3.00 |
| | Sodium tripolyphosphate | 12.0 | 12.0 | 12.00 | 12.00 |
| 25 | PEG 20,000 | 2.0 | 2.0 | 2.00 | 2.00 |
| | Potassium persulfate | 26.0 | 26.0 | 26.00 | 26.00 |
| | Sodium carbonate | 2.0 | 2.0 | 2.00 | 2.00 |
| | Pyrogenic silica | 2.0 | 2.0 | 2.00 | 2.00 |
| | Dye/flavor | 2.0 | 2.0 | 2.00 | 2.00 |

30 In Examples 62-65 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 62-65, any combination of the protease nzymes useful in the present invention recited in Tables III, V and

35 VI, among others, are substituted for Protease # 12 with substantially similar results.

3. Personal Cleansing Compositions

In another embodiment of the present invention, personal cleaning compositions for cleaning the skin (in liquid and bar form) comprise one or more of the protease enzymes. Such compositions typically comprise from about 0.001% to about 5% protease enzyme, preferably from about 0.005% to about 2%, and most preferably from about 0.01% to about 0.8% by weight of the composition. Preferred personal cleansing compositions into which can be included protease enzymes as described herein are taught in U.S. Patent Applications SN 08/121,623 and SN 08/121,624, both by Visscher et al., filed September 14, 1993, the disclosures of which are incorporated herein by reference in their entirety. Such compositions are illustrated by the following examples.

Example 66

Liquid Personal Cleansing Compositions Containing Soap

| | <u>Component</u> | <u>Weight %</u> |
|----|-----------------------------------|---------------------------|
| 15 | Soap (K or Na) | 15.00 |
| | 30% Laurate | |
| | 30% Myristate | |
| | 25% Palmitate | |
| | 15% Stearate | |
| 20 | Fatty acids (above ratios) | 4.50 |
| | Na Lauryl Sarcosinate | 6.00 |
| | Sodium Laureth-3 Sulfate | 0.66 |
| | Cocamidopropylbetaine | 1.33 |
| | Glycerine | 15.00 |
| 25 | Propylene glycol | 9.00 |
| | Polyquaternium 10 | 0.80 |
| | Ethylene glycol distearate (EDTA) | 1.50 |
| | Propylparaben | 0.10 |
| | Methylparaben | 0.20 |
| 30 | Protease # 12 | 0.10 |
| | KOH or NaOH | If necessary to adjust pH |
| | Calcium sulfate | 3 |
| | Acetic acid | 3 |
| | Water | Balance to 100 |

35

Example 67

Personal Cleansing Bar Composition

| <u>Component</u> | <u>Weight %</u> |
|------------------|-----------------|
|------------------|-----------------|

| | | |
|----|---------------------------------|----------------|
| | Sodium Cocoyl Isethionate | 47.20 |
| | Sodium Cetearyl Sulfate | 9.14 |
| | Paraffin | 9.05 |
| | Sodium Soap (in situ) | 3.67 |
| 5 | Sodium Isethionate | 5.51 |
| | Sodium Chloride | 0.45 |
| | Titanium Dioxide | 0.4 |
| | Trisodium EDTA | 0.1 |
| | Trisodium Etidronate | 0.1 |
| 10 | Perfume | 1.20 |
| | Na ₂ SO ₄ | 0.87 |
| | Protease # 12 | 0.10 |
| | Water/Minors | Balance to 100 |

In Examples 66-67 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 66-67, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

Example 68

Wash Performance Test

The wash performance of the variants useful in the present invention compositions is evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030).

Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, are placed in each pot of a Model 7243S Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness (Ca⁺⁺:Mg⁺⁺::3:1::w:w), 7 g of detergent, and enzyme as appropriate. The detergent base is WFK1 detergent from wfk - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

| Component | % of Final Formulation |
|------------------------------|------------------------|
| Zeolite A | 25% |
| Sodium sulfate | 25% |
| Soda Ash | 10% |
| Linear alkylbenzenesulfonate | 8.8% |

| | |
|--|------|
| Alcohol ethoxylate (7-8 EO) | 4.5% |
| Sodium soap | 3% |
| Sodium silicate (SiO ₂ :Na ₂ O::3.3:1) | 3% |

To this base detergent, the following additions are made:

| Component | % of Final Formulation |
|------------------------------|------------------------|
| Sodium perborate monohydrate | 13% |
| Copolymer (Sokalan CP5) | 4% |
| TAED (Mykon ATC Green) | 3% |
| Enzyme | 0.5% |
| Brightener (Tinopal AMS-GX) | 0.2% |

- 5 Sodium perborate monohydrate is obtained from Degussa Corporation, Ridgefield-Park, NJ 07660. Sokalan CP5 is obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetythylenediamine) is obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX is obtained from Ciba-Geigy Corporation, Greensboro, NC 27419.

- 10 Six EMPA 116 swatches are washed in detergent with enzyme for 30 min at 60°C and are subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes are added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses. Swatches are dried and pressed, and the reflectance from the swatches is measured using the L value on the L*a*b* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. lentus* (GG36) protease and is calculated by dividing the amount of *B. lentus* (GG36) protease by the amount of variant protease that is needed to provide the same stain removal performance X 100. The data are shown in Table VII.

Table VII

| Enzyme | Wash Performance |
|-----------------------------|------------------|
| <i>B. lentus</i> subtilisin | 100 |
| N76D | 310 |
| N76D/S103A | 230 |
| N76D/V104I | 130 |
| N76D/V107V | 160 |
| N76D/S99D/S101R | 370 |
| N76D/S99D/S103A | 290 |
| N76D/S101R/S103A | 130 |
| N76D/S101R/V104I | 300 |
| N76D/S103A/V104I | 320 |
| N76D/S103G/V104I | 160 |
| N76D/S103A/V104F | 210 |
| N76D/S103A/V104N | 110 |

| | |
|------------------------|-----|
| N76D/S103A/V104T | 170 |
| N76D/V104I/I107V | 210 |
| N76D/S99D/S101R/S103A | 220 |
| N76D/S99D/S101R/V104I | 140 |
| N76D/S101G/S103A/V104I | 170 |
| N76D/S101R/S103A/V104I | 150 |
| N76D/S103A/V104I/S105A | 170 |
| N76D/S103A/V104T/I107A | 120 |
| N76D/S103A/V104T/I107L | 110 |
| N76D/S103A/V104I/L126F | 110 |
| N76D/S103A/V104I/S128G | 280 |
| N76D/S103A/V104I/L135I | 160 |
| N76D/S103A/V104I/L135V | 160 |
| N76D/S103A/V104I/D197E | 170 |
| N76D/S103A/V104I/N204A | 160 |
| N76D/S103A/V104I/N204G | 150 |
| N76D/S103A/V104I/P210I | 470 |
| N76D/S103A/V104I/M222A | 100 |
| N76D/S103A/V104I/T260P | 280 |
| N76D/S103A/V104I/S265N | 190 |

Example 69

Protease Stability in a Liquid Detergent Formulation

- A comparison of protease stability toward inactivation in a liquid detergent formulation is made for *Bacillus lentus* subtilisin and its variant enzyme N76D/S103A/V104I according to the procedure outlined herein. The detergent formulation for the study is a commercially purchased bottle of Tide Ultra liquid laundry detergent made in the USA by The Procter & Gamble Company. Heat treatment of the detergent formulation is necessary to inactivate *in-situ* protease. This is accomplished by incubating the detergent at 96°C for a period of 4.5 hours. Concentrated preparations of the *B. lentus* subtilisin and N76D/S103A/V104I variant, in the range of 20 grams/liter enzyme, are then added to the heat-treated Tide Ultra at room-temperature to a final concentration of 0.3 grams/liter enzyme in the detergent formulation. The heat-treated detergent with protease added is then incubated in a water bath thermostatted at 50°C. Aliquots are removed from the incubation tubes at 0, 24, 46, 76, and 112 hour time intervals and assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate suc-Ala-Ala-Pro-phe-p-nitroanilide dissolved in 0.1M tris-HCL buffer, pH 8.6, and thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410nm as a function of time. As shown in Fig. 10, the N76D/S103A/V104I variant is observed to have significantly greater stability towards inactivation than the native *B. lentus* enzyme. Estimated half-lives for inactivation in the Tide Ultra detergent formulation for the two enzymes, under

the specified test conditions, at 45 hours for *B. lentus* subtilisin and 125 hours for the N76D/S103A/V104I variant.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and
5 modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Baeck, Andre (NMN)
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Wilson, Lori J.
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Barnett, Bobby L.
Rubingh, Donn N.
- (ii) TITLE OF INVENTION: Protease-Containing Cleaning Compositions
- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: The Procter & Gamble Company
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 - (F) ZIP: 45253-8707
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 13-OCT-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGCTGCAA CTCGTAAA

19

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
GCTGCTCTAG ACAATTCG 18
- (2) INFORMATION FOR SEQ ID NO:3:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
GTATTAGGGG CGGACGGTCG AGGCGCCATC AGCTCGATT 39
- (2) INFORMATION FOR SEQ ID NO:4:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
TCAGGTTCGG TCTCGAGCGT TGCCCAAGGA TTG 33
- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CACGTTGCTA GCTTGAGTTT AG 22
- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1497 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
GGTCTACTAA AATATTATTC CATACTATAC AATTAATACA CAGAATAATC TGTCTATTGG 60
TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA 120

GTTTGCTGTT TGCTTTAGCG TTAATCTTTA CGATGGCGTT CGGCAGCACA TCCTCTGCCC 180
 AGGCGGCAGG GAAATCAAAC GGGGAAAAGA AATATATTGT CGGGTTTAAA CAGACAATGA 240
 GCACGATGAG CGCCGCTAAG AAGAAAGATG TCATTTCTGA AAAAGGCGGG AAAGTGCAAA 300
 AGCAATTCAA ATATGTAGAC GCAGCTTCAG TCACATTAAA CGAAAAAGCT GTAAAAGAAT 360
 TGAAAAAGA CCCGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC 420
 AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCGTC TCTGCACTCT CAAGGCTACA 480
 CTGGATCAAA TGTTAAAGTA GCGGTTATCG ACAGCGGTAT CGATTCTTCT CATCCTGATT 540
 TAAAGGTAGC AAGCGGAGCC AGCATGGTTC CTTCTGA AAC AAATCCTTTC CAAGACAACA 600
 ACTCTCACGG AACTCACGTT GCCGGCACAG TTGCGGCTCT TAATAACTCA ATCGGTGTAT 660
 TAGGCGTTGC GCCAAGCGCA TCACTTTACG CTGTAAAAGT TCTCGGTGCT GACGGTTCCG 720
 GCCAATACAG CTGGATCATT AACGGAATCG AGTGGGCGAT CGCAAACAAT ATGGACGTTA 780
 TTAACATGAG CCTCGGCGGA CCTTCTGGTT CTGCTGCTTT AAAAGCGGCA GTTGATAAAG 840
 CCGTTGCATC CGGCGTCGTA GTCGTTGCGG CAGCCGGTAA CGAAGGCACT TCCGGCAGCT 900
 CAAGCACAGT GGGCTACCCT GGTAAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA 960
 GCAGCAACCA AAGAGCATCT TTCTCAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG 1020
 GCGTATCTAT CCAAAGCAGC CTTCTGGAA ACAAAATACGG GGCGTACAAC GGTACGTCAA 1080
 TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA 1140
 CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT 1200
 ACTATGGAAA AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAAACATA AAAAACCAGC 1260
 CTTGGCCCCG CCGGTTTTTT ATTATTTTTC TTCCTCCGCA TGTTCAATCC GCTCCATAAT 1320
 CGACGGATGG CTCCCTCTGA AAATTTTAAC GAGAAACGGC GGGTTGACCC GGCTCAGTCC 1380
 CGTAACGGCC AACTCCTGAA ACGTCTCAAT CGCCGCTTCC CGGTTTCCGG TCAGCTCAAT 1440
 GCCATAACGG TCGGCGGCGT TTTCCTGATA CCGGGAGACG GCATTCTGTA TCGGATC 1497

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
 1 5 10 15
 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
 20 25 30
 Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
 35 40 45

60

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
 50 55 60
 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125
 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
 130 135 140
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
 145 150 155 160
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
 165 170 175
 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
 180 185 190
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
 210 215 220
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
 225 230 235 240
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
 245 250 255
 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 275 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu
 1 5 10 15
 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
 20 25 30
 Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala
 35 40 45
 Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His
 50 55 60
 Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80

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Val Leu Gly Val Ser Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125
 Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser
 130 135 140
 Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
 145 150 155 160
 Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala
 165 170 175
 Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala
 180 185 190
 Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr
 210 215 220
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr
 225 230 235 240
 Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr
 245 250 255
 Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 274 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val
 1 5 10 15
 Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala
 35 40 45
 Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly
 50 55 60
 Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val
 65 70 75 80
 Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys Val Leu Asn
 85 90 95

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Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly Ile Glu Trp
 100 105 110
 Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu Gly Gly Ala
 115 120 125
 Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala Tyr Ala Arg
 130 135 140
 Gly Val Val Val Val Ala Ala Ala Gly Asn Ser Gly Asn Ser Gly Ser
 145 150 155 160
 Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val Ile Ala Val
 165 170 175
 Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser Ser Val Gly
 180 185 190
 Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr Ser Thr Tyr
 195 200 205
 Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met Ala Ser Pro
 210 215 220
 His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu
 225 230 235 240
 Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu
 245 250 255
 Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala
 260 265 270
 Ala Gln

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala
 1 5 10 15
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
 35 40 45
 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
 50 55 60
 His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
 65 70 75 80
 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
 85 90 95
 Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
 100 105 110

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Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
 115 120 125
 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
 130 135 140
 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
 145 150 155 160
 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
 165 170 175
 Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
 180 185 190
 Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
 195 200 205
 Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
 210 215 220
 Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
 225 230 235 240
 Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
 245 250 255
 Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
 260 265

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

| | |
|--|-----|
| ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT | 60 |
| AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAGAAA AATATTTAAT TGGCTTTAAT | 120 |
| GAGCAGGAAG CTGTCACTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT | 180 |
| CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT | 240 |
| TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTCTT | 300 |
| TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAAATTAGC | 360 |
| CGTGTGCAAG CCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT | 420 |
| GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT | 480 |
| GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG | 540 |
| ATTGCTGCTT TAAACAATTC GATTGGCGTT CTTGGCGTAG CQCCGAGCGC GGAAGTATAC | 600 |
| GCTGTTAAAG TATTAGGGGC GAGCGGTTCA GGTTCGGTCA GCTCGATTGC CCAAGGATTG | 660 |
| GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTGA GTTTAGGAAG CCCTTCGCCA | 720 |
| AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG | 780 |
| GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG | 840 |

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GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900
 CTTGACATTG TCGCACCAGG TGTAACGTG CAGAGCACAT ACCCAGGTTT AACGTATGCC 960
 AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CTTGTGTTAA 1020
 CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080
 AGCTTAGGAA GCACGAACTT GTATGGAAGC GGAATTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1140 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCACAC TACTCATTTT TGTGCTTTT 60
 AGTTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT 120
 GAGCAGGAAG CTGTCAGTGA GTTGTAGAA CAAGTAGAGG CAAATGACGA GGTGCGCCATT 180
 CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT 240
 TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTTCT 300
 TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGAATTAGC 360
 CGTGTGCAAG CCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420
 GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480
 GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540
 ATTGCTGCTT TAGACAATC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACATAC 600
 GCTGTTAAAG TATTAGGGGC GAGCGGTTC GCGCCCATCA GCTCGATTGC CCAAGGATTG 660
 GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTGA GTTTAGGAAG CCCTTCGCCA 720
 AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTGTAGCG 780
 GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840
 GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900
 CTTGACATTG TCGCACCAGG TGTAACGTG CAGAGCACAT ACCCAGGTTT AACGTATGCC 960
 AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CTTGTGTTAA 1020
 CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080
 AGCTTAGGAA GCACGAACTT GTATGGAAGC GGAATTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATGCCAGCC ACAACGGTAC TTCGATGGCT

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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T

31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT

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What is claimed is:

1. A cleaning composition comprising:

- (a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin; and**
- (b) one or more cleaning composition materials compatible with the protease enzyme.**

2. The cleaning compositions according to Claim 1 wherein the cleaning composition materials are selected from the group consisting of surfactants, solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, builders, bleaching agents, dyes, perfumes, and mixtures thereof.

3. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least about 1% surfactant by weight of the composition, said surfactant comprising materials selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxyates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof.

4. The cleaning composition according to Claim 3 further comprising at least about 5% builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

5. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one bleaching agent.
6. The cleaning compositions according to Claim 5 wherein the bleaching agent is selected from the group consisting of percarbonates, perborates, and mixtures thereof, and optionally further comprising at least one bleach activator.
7. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases and mixtures thereof.
8. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one fabric softener.
9. The cleaning compositions according to Claim 1 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of substitutions is made at the positions equivalent to 76/99, 76/101, 76/103, 76/104, 76/107, 76/123,
5 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/126, 76/103/104/135, 76/103/104/197, 76/103/104/222,
10 76/103/104/260, 76/103/104/265, 76/103/104/126/265, 27/76/104/123/274, 27/76/104/109/123/274, 27/76/104/123/218/274, 27/76/104/123, 27/76/104/107/123, 27/76/104/109/123, 27/76/104/109/123/218/274, 27/76/104/123/197, 27/76/104/123/204, 27/76/104/123/206, 27/76/104/123/216, 27/76/104/123/218, 27/76/104/123/260,
15 27/76/104/123/195/197, 27/76/104/123/195/218, 27/76/104/123/197/218, 27/76/104/123/204/218, 27/76/104/123/206/218, 27/76/104/123/218/260, 27/76/104/123/195/197/218, 76/103/104/217, 76/103/104/156, 76/103/104/166, 76/103/104/105, 76/101/103/104, 76/103/104/128, 76/103/104/210, 76/103/104/107, 76/103/104/204, 76/217, 76/103/104/156/166 and
20 76/103/104/128.

10. The cleaning composition according to Claim 9 where in the protease enzyme is a subtilisin variant selected from the group consisting of 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222.

11. A fabric cleaning composition comprising:

- (a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N and N76D/S103A/V104I/M222A and mixtures thereof; and
- (b) one or more cleaning composition materials compatible with the protease enzyme comprising at least about 5% surfactant and at least about 5% builder, by weight of the composition.

12. The fabric cleaning composition according to Claim 11 further comprising cleaning composition materials selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds

boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

13. The fabric cleaning composition according to Claim 11 further comprising at least one bleaching agent.

14. The fabric cleaning composition according to Claim 11 further comprising at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases, and mixtures thereof.

15. The fabric cleaning composition according to Claim 11 in the form of a liquid, granule or bar.

16. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a subtilisin variant selected from N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V, N76D/S101R/S103A/V104I,
5 N76D/S99D/S101R/S103A/V104I, N76D/S101R/V104I, and mixtures thereof.

17. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a Bacillus subtilisin variant selected from N76D/V104I, N76D/S103A/V104I, and mixtures thereof.

18. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from Bacillus lentus subtilisin;

(b) at least about 5% surfactant;

(c) at least about 5% builder; and

(d) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds
10 boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

19. The fabric cleaning composition according to Claim 18 wherein the surfactant is selected from the group consisting of alkyl benzen sulfonates,

primary-alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxyates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof; and wherein further the builder is selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

20. The fabric cleaning composition according to Claim 19 further comprising one or more cleaning composition materials selected from the group consisting of bleaching agents, fabric softeners, and enzymes.

21. The fabric cleaning composition according to Claim 18 in the form of a concentrated granular fabric cleaning composition comprising at least about 30% surfactant.

22. A dishwashing composition comprising:

- (a) from about 0.0001% to about 10% protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and N76D/S103A/V104I/M222A and mixtures thereof;
- (b) from about 0.1% to about 10% surfactant; and

(c) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, dispersing agents, suds suppressors, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

23. The dishwashing composition according to Claim 22 wherein the protease enzyme is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

24. A personal cleansing composition comprising:

- (a) from about 0.001% to about 5% by weight of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin;
- (b) from about 0.01% to about 95% by weight of a surfactant system; and
- (c) optionally, from about 0.05% to about 50% of an enzyme stabilizer.

25. The composition of Claim 24 wherein the protease enzyme is at a level of from about 0.001% to about 2% by weight.

26. The composition of Claim 25 wherein the protease enzyme is at a level of from about 0.01% to about 0.8% by weight.

27. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of anionic carboxylates, amine oxides, alkyl glucosides, glucose amides, alkyl sulfates, alkyl ether sulfates, acyl isethionates, alkyl sulfosuccinates, alkyl phosphate esters, ethoxylated phosphate esters, alkyl glyceryl ether sulfonates, or mixtures thereof.

28. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of soaps, acylglutamates, alkyl

sarcosinates, lauramine oxides, cocamine oxides, cocamidopropylamine oxides, decylglucosides, lauryl sulfates, laureth sulfates, C₁₂-C₁₈ acyl isethionates, or mixtures thereof.

29. The composition of Claim 28 wherein the surfactant is soap at a level of at least about 2% by weight of the composition.

30. The composition of Claim 29 wherein the soap is at a level of at least about 10% by weight of the composition.

31. The composition of Claim 30 wherein the soap is at a level of at least about 25% by weight of the composition.

32. The composition of Claim 28 wherein the ratio of soap to protease enzyme is from about 2,000:1 to about 8:1.

33. The composition of Claim 32 wherein the ratio of soap to protease enzyme is from about 400:1 to about 40:1.

34. The composition of Claim 28 wherein the protease enzyme is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D;
 5 N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S;
 N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I;
 N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I;
 N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;
 N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;
 10 N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I;
 N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S;
 N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S;
 N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;
 15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N;
 N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A;
 N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F;
 N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and
 N76D/S103A/V104I/M222A; and mixtures thereof.

35. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

36. A method for cleaning dishes, said method comprising contacting a dish in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

37. A method for personal cleansing, said method comprising contacting the part of the human or lower animal body in need of cleaning with a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the
5 protease enzyme is a subtilisin variant selected from N76D/S99D;
N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S;
N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I;
10 N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I;
N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;
N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;
N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I;
15 N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S;
N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S;
N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;
20 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N;
N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A;
N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F;
25 N76D/S103A/V104I/L107T; N76D/S103A/V104I/L126F/S265N and
N76D/S103A/V104I/M222A; and mixtures thereof.

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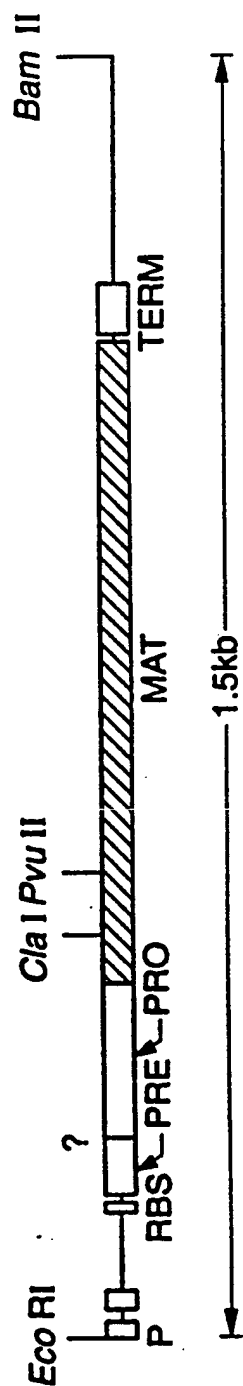


FIG. 1A

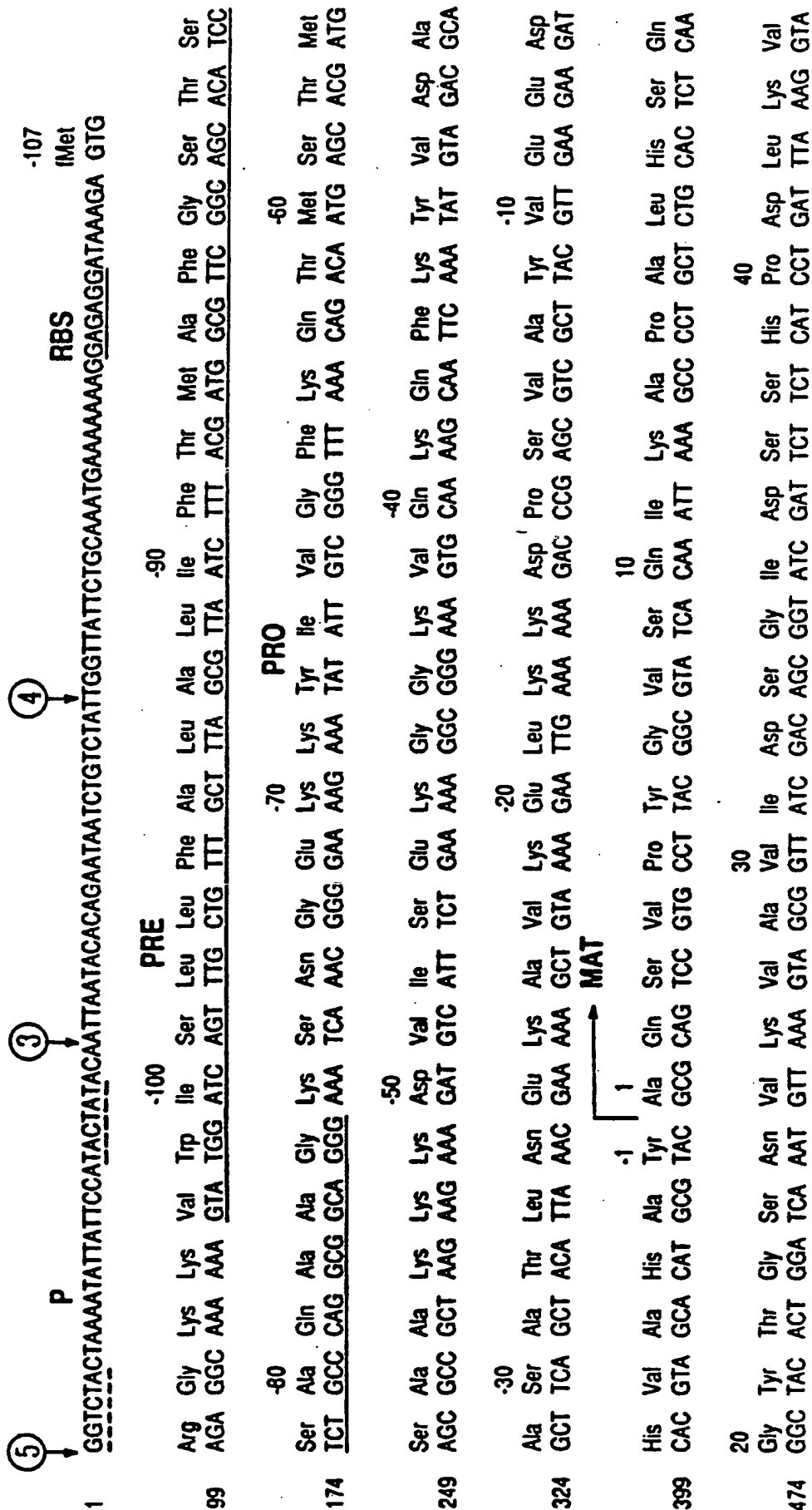


FIG. 1B - 1

549 Ala Gly Gly Ala Ser Met Val Val Pro Ser Gly Thr Asn Pro Phe Gln Asp Asp 60 Asp
 GCA GGC GGA GCC AGC ATG GTT CCT TCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC Gly Thr His Val Ala
 GCC

624 Thr Val Val Ala Leu Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser 90
 GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA

699 Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met
 GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG

774 Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Val Ala Val Ala
 GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA

849 Ser Gly Val Val Val Val Val Ala Ala Gly Asn Gly Thr Ser Gly Thr Ser Ser Thr Val Gly Tyr Pro Gly
 TCC GGC GTC GTA GTC GTT GGT AAC AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT

924 Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro
 AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

999 Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly
 GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

1074 Thr Ser Met Ala Ser Pro His Val Val Ala Gly Ala Ala Leu Ile Leu Ser Lys His Pro Asn Thr Asn Thr
 ACG TCA ATG GCA TCT CCG CAC GGT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

FIG. 1B - 2

1149 Gln Val Arg Ser Ser Leu Gln Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
 CAA GTC CGC AGC AGT TTA GAA AAC AAC ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC
 250 Gln
 1224 GTA CAG CCG GCA GCT CAG TAA AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTATTTTTTCTTCCTCCGCAATGTTCAATCCGCTCC
 270 Val Gln Ala Ala Ala Gln OC
 1224 GTA CAG CCG GCA GCT CAG TAA AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTATTTTTTCTTCCTCCGCAATGTTCAATCCGCTCC

TERM

1316 ATAATCGACGGATGGCTCCCTCTGAAAAATTTAACGAGAAACGGCGGTTGACCCGGCTCAGTCCCGTAACGGCCAAAGTCCTGAAACGTCCTCAATCGCCG

1416 CTCCCGGTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGCGTTTTCCTGATACCGGGGAGACGGCATTCGIAATCGGATC

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FIG._1B - 3

FIG._1B - 1

FIG._1B - 2

FIG._1B - 3

FIG._1B

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CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

| | | |
|---|---------------|-----|
| 1 | 10 | 20 |
| A Q S V P . G | A P A . H . . | G |
| 21 | 30 | 40 |
| . T G S . V K V A V . D . G | H P | |
| 41 | 50 | 60 |
| D L . . . G G A S . V P | Q D | |
| 61 | 70 | 80 |
| . N . H G T H V A G T . A A L N N S I G | | |
| 81 | 90 | 100 |
| V L G V A P S A . L Y A V K V L G A . G | | |
| 101 | 110 | 120 |
| S G . . S . L . . G . E W A . N | | |
| 121 | 130 | 140 |
| V . N . S L G . P S . S | A . . | |
| 141 | 150 | 160 |
| G V . V V A A . G N . G . . . | | |
| 161 | 170 | 180 |
| Y P . . Y A V G A . | | |
| 181 | 190 | 200 |
| D . . N . . A S F S . . G . . L D . . A | | |
| 201 | 210 | 220 |
| P G V . . Q S T . P G . . Y . . . N G T | | |
| 221 | 230 | 240 |
| S M A . P H V A G A A A L . . . K . . . | | |
| 241 | 250 | 260 |
| W . . . Q . R . . L . N T . . . L G . . | | |
| 261 | 270 | |
| . . Y G . G L . N . . A A . . | | |

FIG._2

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COMPARISON OF SUBTILISIN SEQUENCES FROM:

*B.amyloliquefaciens**B.subtilis**B.licheniformis**B.lentus*

| | | | | |
|---|-----|-----|-----|--|
| 01 | 10 | 20 | 30 | |
| A Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P | | | | |
| A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P | | | | |
| A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P | | | | |
| A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P | | | | |
| 41 | 50 | 60 | 70 | |
| D D L K V A G G A S M V P S E E T N P F Q D N N S H G T H V A G T V A A L N N S I G | | | | |
| D D L N V R G G A S F V P S E E T N P F Y Q D D G S S H G T H V A G T V A A L N N S I G | | | | |
| D D L N V V G G A S F V P A G E A Y N * T D D G N G H G T H V A G T V A A L N N S I G | | | | |
| D D L N I R G G A S F V P G E * P S T Q D D G N G H G T H V A G T V A A L N N S I G | | | | |
| 81 | 90 | 100 | 110 | |
| V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D | | | | |
| V L G V S P S A S L Y A V K V L D S T G S G Q Y S W I I N G I E W A I S N N M D | | | | |
| V L G V A P S V S L Y A V K V L N S S G S G Q Y S G I I V S G I E W A I T N N M D | | | | |
| V L G V A P S A E L Y A V K V L G A S G S G Q Y S S I A Q G L E W A G N N G M H | | | | |
| 121 | 130 | 140 | 150 | |
| V I N M S L G G P S G S A A L K A A V D K A V A S G V V V A A A A G N E G T S S G | | | | |
| V I N M S L G G P T G S S T A L K T V V D K A V S S G I V V A A A A G N E G S S G | | | | |
| V I N M S L G G A S S G S T A M K Q A V D N A Y A R G V V V A A A A G N S G S G | | | | |
| V A N L S L G S P S A T L E Q A V N S A T S R G V L V V A A A A G N S G A S | | | | |

FIG.-3A

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[illegible]

FIG. 3B

FIG.-3A

FIG.-3B

FIG. 3

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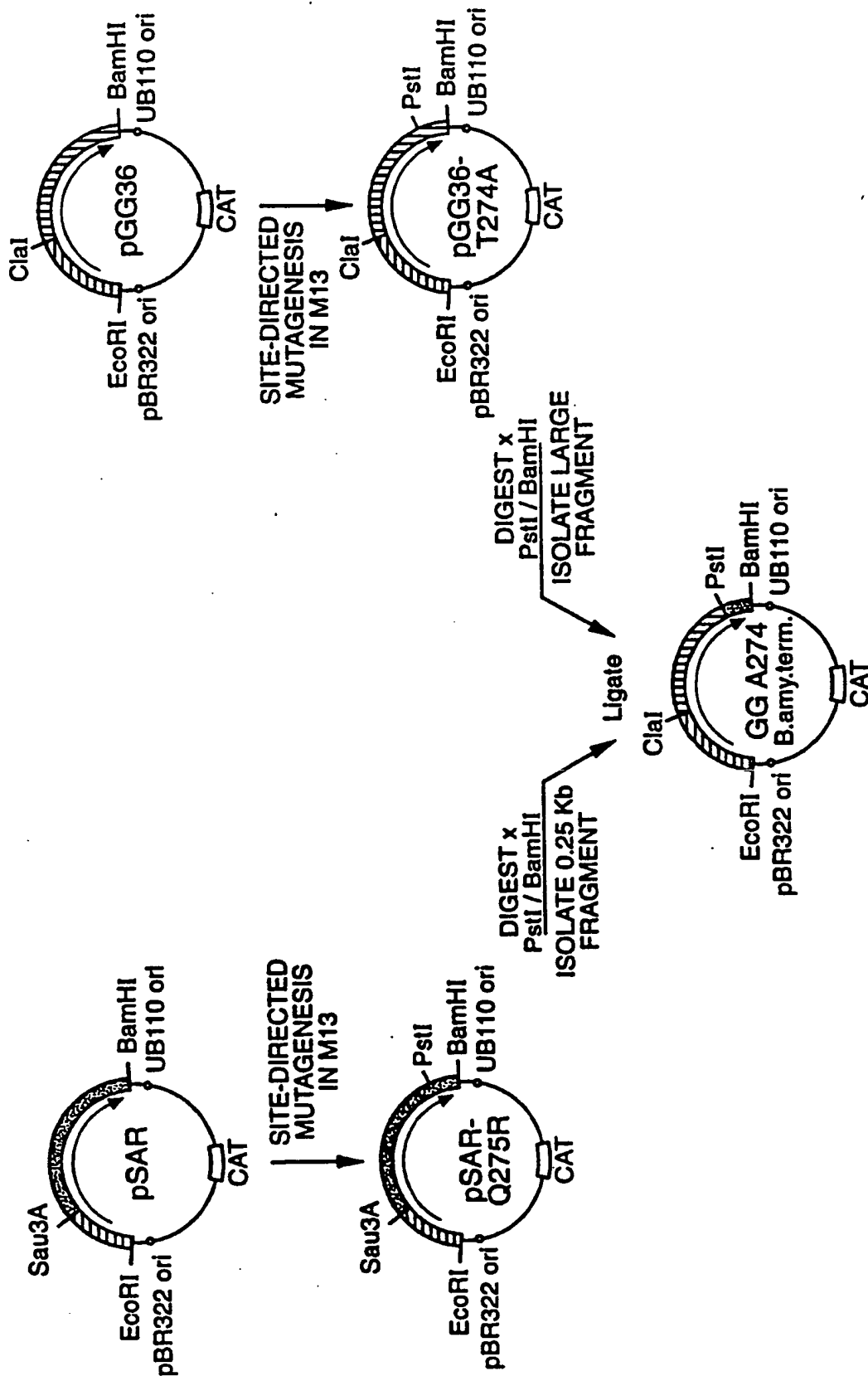
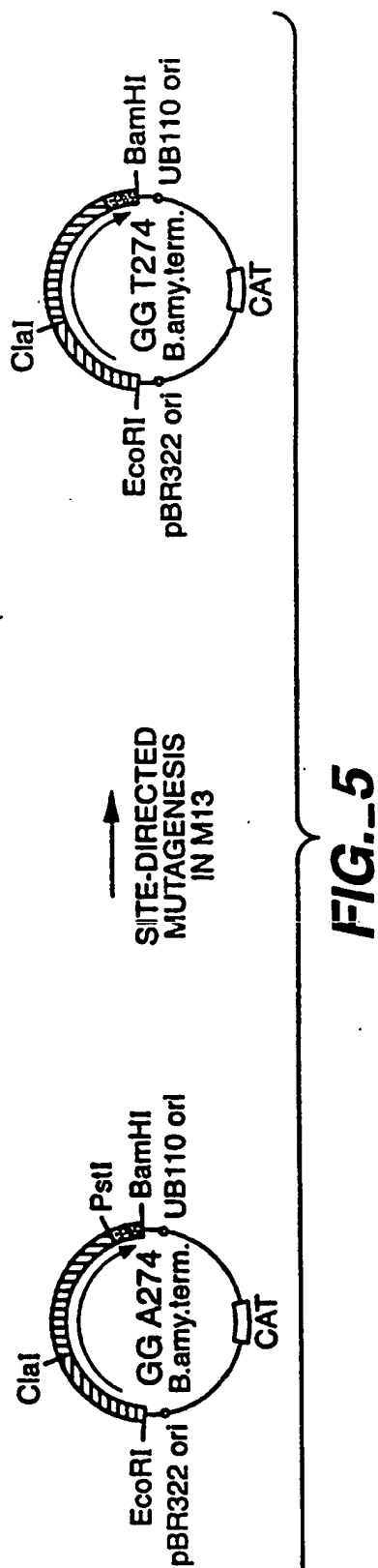


FIG. 4

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10 30 50
ATGAAGAAACCGTTGGGAAATTCGCAAGCACCGCACTACTCATTTCTGTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110
AGTTCATCGATCGCATCGCTGCTGAAGCAAGCAAAATAATTTAATTGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170
GAGCAGGAAGCTGTCAGTGAGTTGTAGAACAAAGTAGAGGCAATGACGAGGTCCGCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230
CTCTCTGAGGAAGAGGAAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290
TTATCCGTTGAGTTAAGCCAGAAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350
TATATTGAAGAGGATGCAGAAAGTAACGACAATGGCGCAATCAGTGCCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProThrPglyIleSer

370 390 410
CGTGTCAAGCCCGAGCTGCCCATAAACCGTGGATTGACAGGTTCTGGGTGTAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

FIG.-6A

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430 450 470
GTCTCGATACAGGATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590
ATTGCTGCTTTAAACAATTTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGGAACTATAC
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650
GCTGTTAAAGTATTAGGGCGAGCGGTTTCAGGTTCCGTCAGCTCGATTGCCAAGGATTG
AlaValIleValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

670 690 710
GAATGGCCAGGGAACAATGGCATGCACGTTGCTAATTTAGGTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMethHisValAlaAsnLeuSerLeuGlySerProSerPro

730 750 770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTCTTGTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

FIG..6B

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850 870 890
 GCAGTCGGAGCTACTGACCAAAACAACCGCGCAGCTTTTCACAGTATGGCGCAGGG
 AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly
 910 930 950
 CTTGACATTGTCGCACCGAGGTGTAACGTGCAGAGCACATACCCAGGTTTCAACGTATGCC
 LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla
 970 990 1010
 AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAA
 S rLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaLeuValLys
 1030 1050 1070
 CAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
 GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr
 1090 1110 1130
 AGCTTAGGAAGCACGAACTTGATGGAAAGCGGACTTGTCATGCAGAGCGGCAACACCG
 SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG._6C

FIG._6A

FIG._6B

FIG._6C

FIG._6

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10 30 50
ATGAAGAAACCGTTGGGAAATTGTGCAAGCACCGCACTACTTCTGTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110
AGTTCATCGATCGGCTGCTGAAGAAGCAAAAGAAAATATTAAATTGGCTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170
GAGCAGGAAGCTGTCAGTGAGTTGTAGAACACAGTAGAGGCAATGACGAGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230
CTCTCTGAGGAAGAGGAGTCGAAATTGAAATTGCTTCATGAATTTGAAACGATTCTGT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290
TTATCCGTTGAGTTAAGCCAGAAAGATGTGACGCGCTTGAACTCGATCCAGCGATTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350
TATATTGACAGGATGCAGAAAGTAACGACAAATGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

370 390 410
CGTGTGCAAGCCCCAGCTGCCCATACCGTGGATTGACAGGTTCTGTGTAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

FIG. 7A

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430 450 470
GTCTCGATACAGGTATTCCACTCATCCAGACTTAATATTCGTGGTGGCGTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590
ATTGCTGCTTTAGACAACCTCGATTGGCGTTCTTGCGTAGCGCCGAGCGGGAATATAC
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650
GCTGTTAAAGTATTAGGGCGAGCGGTTTCAGGGCCCATCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu

670 690 710
GAATGGCAGGGAACAATGGCATGCACGTTGCTAATTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMethHisValAlaAsnLeuSerLeuGlySerProSerPro

730 750 770
AGTGCCACACTTGAGCAAGCTGTAAATAGCGCGACTTCTAGAGGCGTTCTTGTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValAla

790 810 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCCAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

FIG. 7B

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850 870 890
GCAGTCGGAGCTACTGACCAAAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly
910 930 950
CTTGACATTGTCGCACACAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla
970 990 1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCCCTTGTTAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys
1030 1050 1070
CAAAAGAACCCATCTTGGTCCAAATGTACAAATCCGCAATCATCTAAAGAAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr
1090 1110 1130
AGCTTAGGAAGCACGAACTTGATGGAAGCGGACTTGTCATGCAGAAAGCGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG..7C

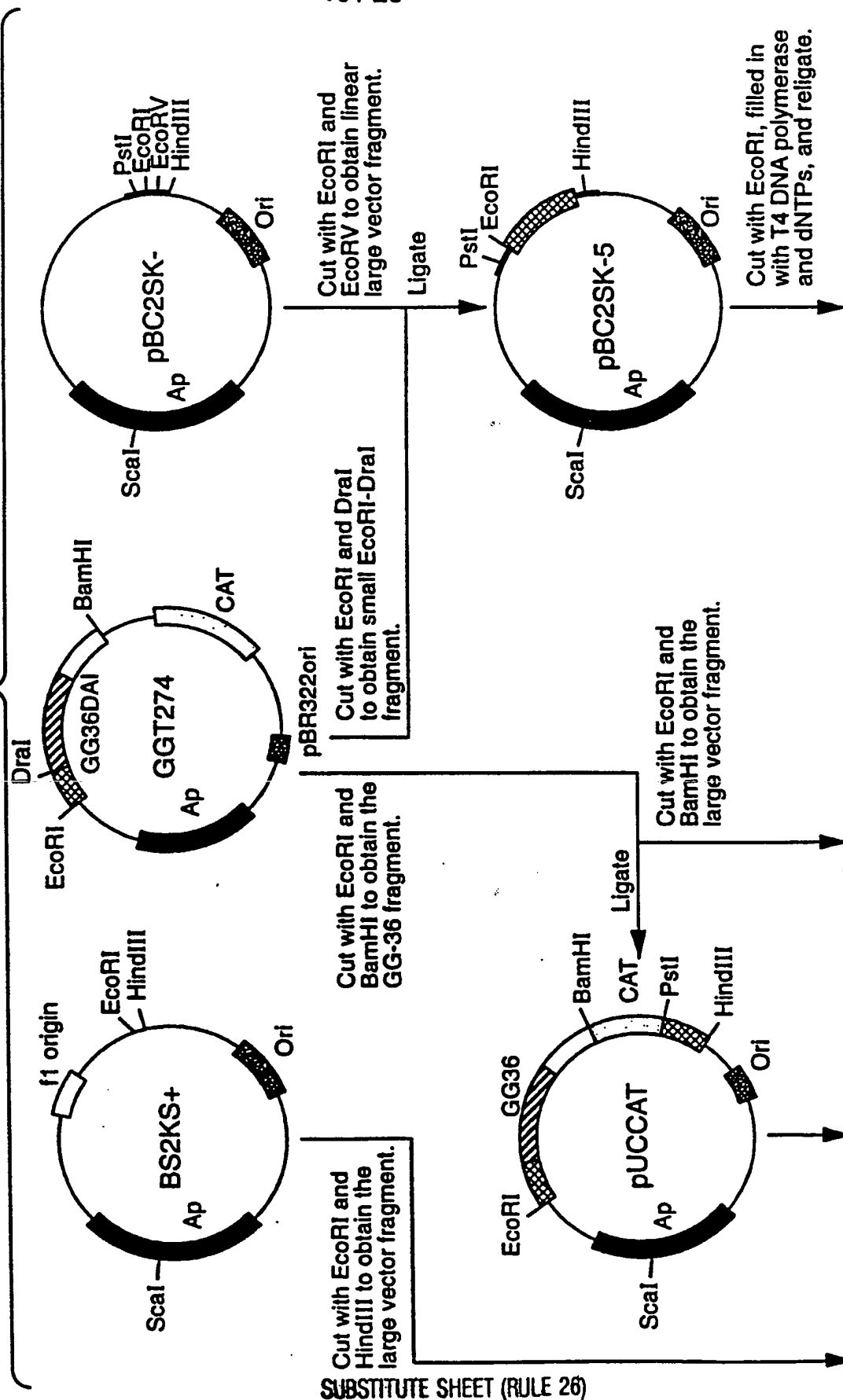
FIG..7

FIG..7A

FIG..7B

FIG..7C

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FIG. 8A

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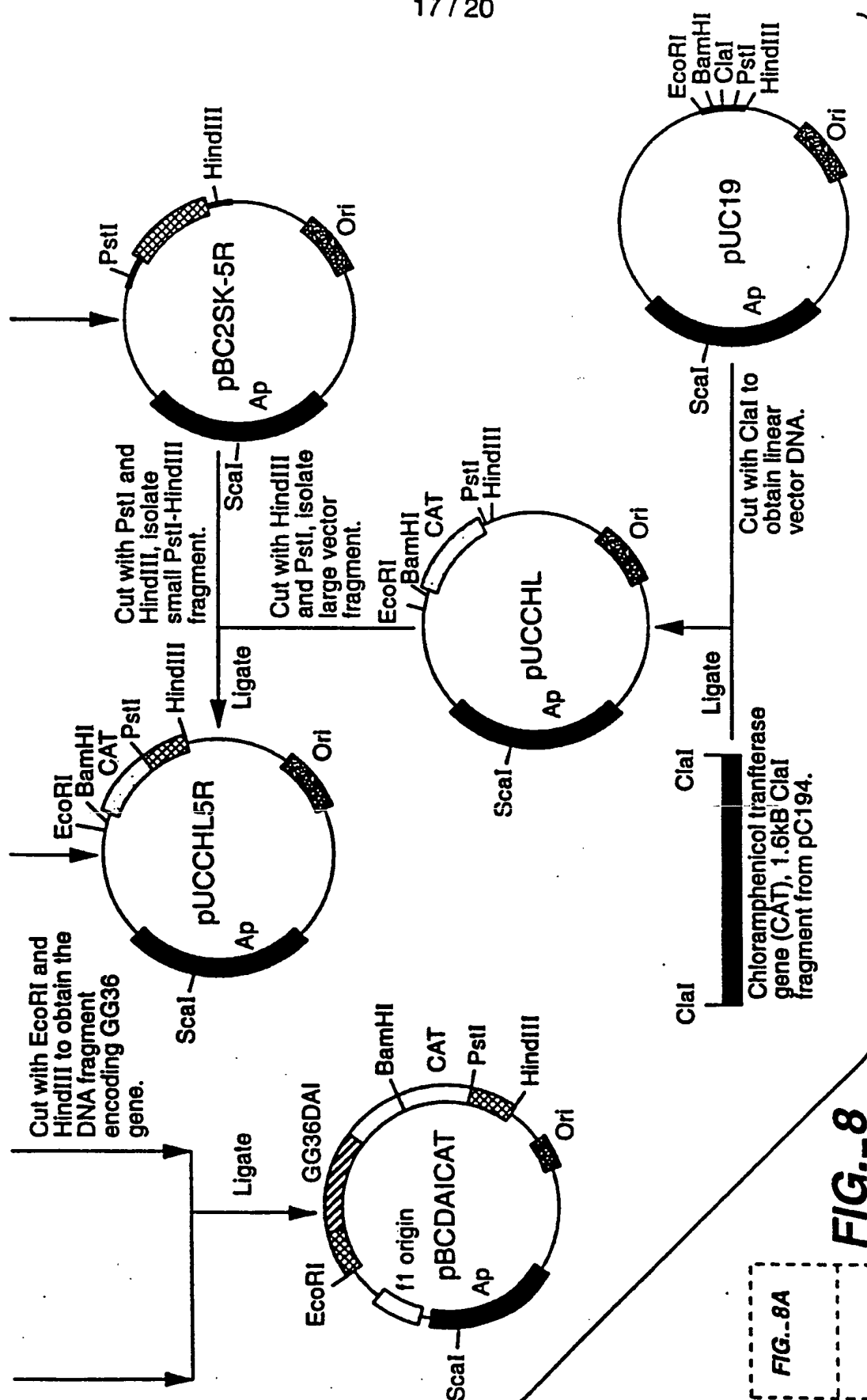


FIG. 8B

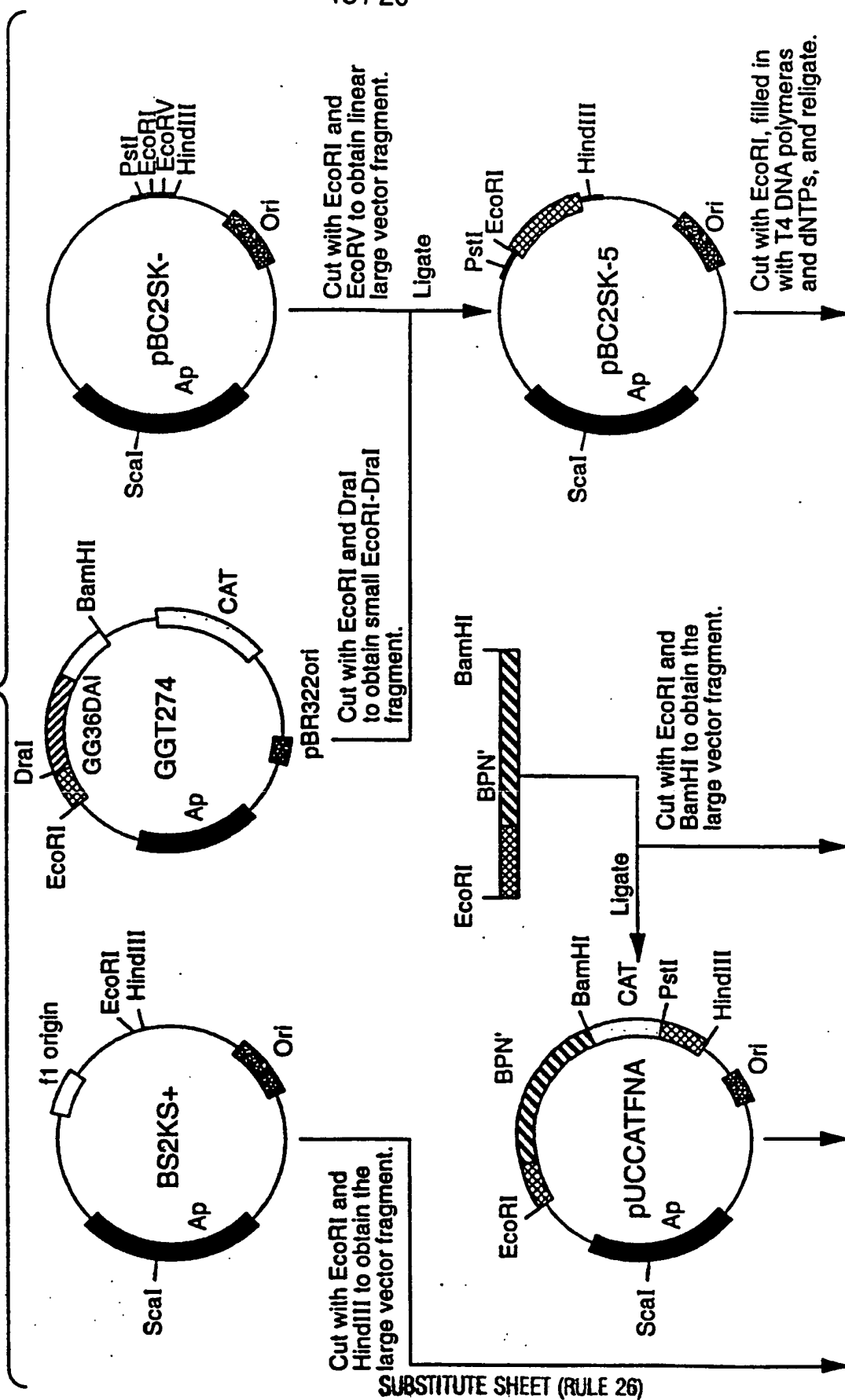
FIG. 8

FIG. 8A

FIG. 8B

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FIG. 9A



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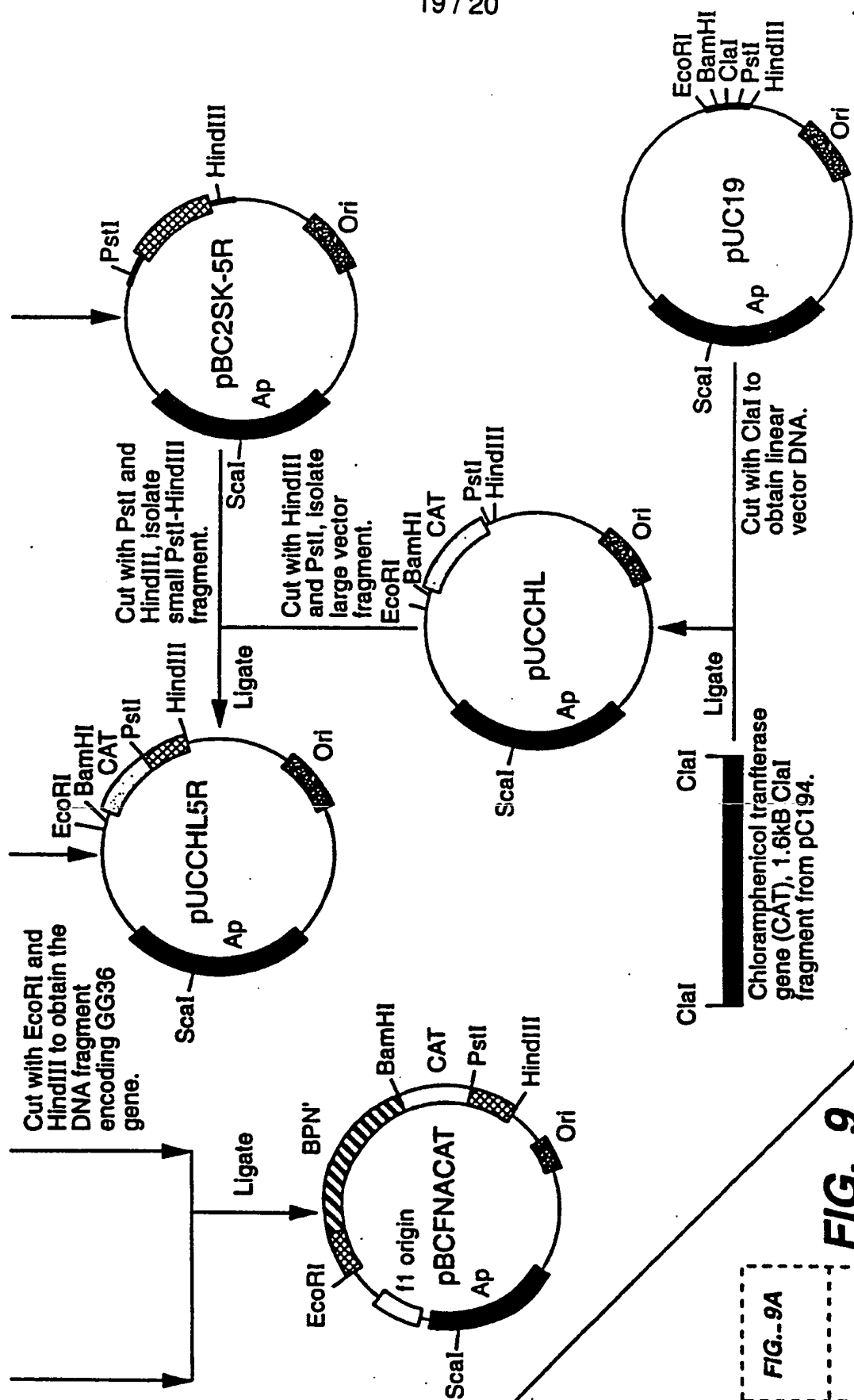


FIG. 9B

FIG. 9

FIG. 9A

FIG. 9B

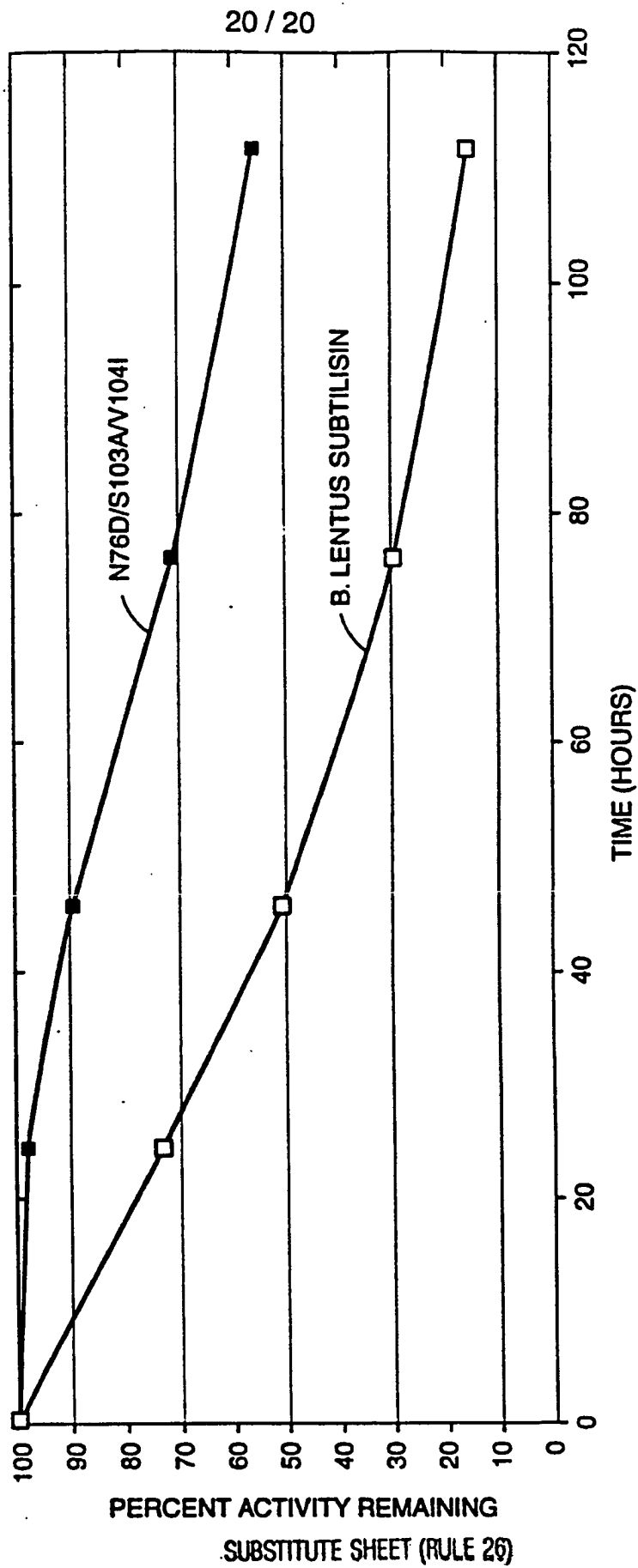


FIG. 10

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 94/11776

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C11D3/386 A61K7/48 C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C11D C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | EP,A,0 398 539 (AMGEN INC.) 22 November 1990 see page 7, line 14 - line 43; claims 1-23; examples | 1-21 |
| X | EP,A,0 405 901 (UNILEVER) 2 January 1991 see page 30, line 25 - page 35, line 47; claims; examples | 1-21 |
| X | WO,A,88 08033 (AMGEN INC.) 20 October 1988 see page 14, line 1 - page 15, line 30; claims | 1-21 |
| X | WO,A,89 09830 (GENEX CORP.) 19 October 1989 see claims 11-16 | 1-21 |
| | --- -/-- | |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

16 February 1995

Date of mailing of the international search report

- 8. 03. 95

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 Fax (+ 31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

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PCT/US 94/11776

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| A | EP,A,0 516 200 (UNILEVER) 2 December 1992 see page 12, line 10 - page 13, line 50; claims 1-10 --- | 1-21 |
| A | WO,A,91 06637 (GENENCOR INT.) 16 May 1991 cited in the application see the whole document --- | 1 |
| A | WO,A,89 06279 (NOVO INDUSTRI) 13 July 1989 see claims ----- | 1 |

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/11776

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